Optimization of Culture Medium Performance for Growth of Microalga *Trachydiscus* sp. LCR-Awa9/2

A thesis submitted in the fulfilment of the requirements for the Degree of Master of Engineering in Chemical and Process Engineering

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ABSTRACT

The native New Zealand microalga *Trachydiscus* sp. LCR-Awa9/2 has potential as a commercial eicosapentaenoic acid (EPA) producer. The aim of this study was to optimize a culture medium to produce a high cell density culture of the microalga in shake flasks incubated at 25˚C. Bold Basal medium (BBM) was used as the basis culture medium for culture media study. The role of major nutrients and ions such as nitrogen, phosphate, chlorine, sulphate, potassium, magnesium and calcium, and trace nutrients such as cobalt, copper, iron, EDTA, citrate, manganese, molybdenum, zinc, selenium, and vitamins was observed. The trace elements study resulted in removing cobalt and boron from the BBM. Supplementing the culture medium with selenium or vitamins did not improve the microalga growth. Additionally, using different sources of iron and chelating agent did not cause significant change in both biomass production and fatty acid profile.

The microalga was able to utilize all nitrogen compounds tested such as sodium nitrate, urea, and ammonium chloride. However, the ammonia culture medium did not produce as high biomass as nitrate and urea culture media. The nitrate culture medium was the most favourable nitrogen source for the microalga in terms of its production of biomass, fatty acid, and EPA.

A dynamic model was developed to determine the nitrogen, phosphorous, and light consumption for the alga population growth. It was found that light limitation occurred in 250 µmol m^{-2} s^{-1} light intensity, therefore the light intensity needed to be increased to 530 µmol m^{-2} s^{-1}. The microalgal productivity was improved 100% when the light intensity was increased to the high light level. The model fitted the data impressively and some unknown parameters were simulated such as maximum growth rate (μ_{max}), consumption ratio (Y), and Monod constant (K). The amount of nitrogen and phosphate needed for the microalga were 0.1113 and 0.0043 g nutrient/g biomass, respectively.

The starvation of nitrogen or phosphorous, and complete starvation (the microalga was cultivated in distilled water) increased the fatty acid production but they stimulated more elaidic acid than EPA.

The final prototype culture medium is called Bold Basal Modified Awarua medium (BBMA). The use of the modified culture medium led to 100% higher biomass production than the media previously used. The highest biomass and EPA production achieved 4.7 g/L dry biomass, with 12% fatty acid of total dry mass and 33.8% EPA as a factor of total fatty acid.

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Chapter I
INTRODUCTION

1.1 Background

Microalgae are a diverse group of organisms that live in a variety of natural habitats (Cooper & Cassie, 1996). They can be found in the hidden depths beneath the surface of the sea, lakes, rivers, ponds and artificial waterways. The individual microalgae are not visible to the naked eye, but can be visible when a significant population develops. Some of the identified species live photosynthetically, while only a few are recognized to grow mixotrophically (using both carbon dioxide and organic carbon as carbon sources) or heterotrophically (using organic carbon sources) (Lee, 2016). Cultivation of microalgae has been being studied for their role in the conversion of CO_2 and as a renewable energy source, food source, and bio-molecule source.

Microalgae have great potentials in the field of renewable sources of fuels and nutraceuticals (Posten, 2013). They are classified as the third biomass generation or "Biomass 3.0", while the first-generation is energy crops for instance palm, and the second generation is agricultural waste. The first generation is a matter of debate because it also serves as a food source, while the second generation is limited in quantities.

Microalgae have some particular qualities that make them attractive (Posten, 2013). First, compared to terrestrial plants, microalgae can produce five times as much biomass per hectare. Second, according to Posten and Walter (2012), microalgae can be grown in arid areas which are unsuitable for agriculture. Third, microalgae contain a high lipid content up to 50% of total dry mass. The microalgae appear simple at coarse scale, and certain properties of their biomass are advantageous industrially. All the biomass from them can be harvested and processed. This form can make the process much more efficient when compared to higher plants, where only a few parts contain oil. Also, pigments like carotenoids can be produced with high quality and quantity. Furthermore, many species of microalgae do not form lignin which is difficult to separate. It makes them easily processed to be used as a source of energy or for other chemical needs.

As well as renewable energy, microalgae also have potential as nutraceutical raw material (Posten, 2013). According to Packer, Harris, and Adams (2016), “Nutraceuticals are natural health product including food or processed food products that have been proved to have medical benefits and can be used without a prescription.” Algae are suitable as natural producers of health products because they have components such as omega-3 fatty acids, and some pigments for instance β carotene and astaxanthin which are elusive in common dietary sources.

Microalgae are a source of polyunsaturated fatty acids (PUFAs) in the food chain and thus advised as a provider of essential fatty acids (Griffiths, Harrison, Smit, & Maharajh, 2016). They are also the source of almost all essential vitamins and pigments such as chlorophyll,

carotenoids, and phycobiliproteins. PUFAs, including omega-3 fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA), are renowned for their nutritional importance (Griffiths et al., 2016). They provide flexible properties and selective permeability of cell membranes, which is vital for brain development, and beneficial to the cardiovascular system. Additionally, according to Packer et al. (2016), "they have health benefits with few if any side effects." Most vertebrates especially humans and mammals do not produce the enzyme to form a double bond in position n-3 and n-6 fatty acid carbon chain despite the fact that they have various of biosynthetic pathways to analyse and synthesize lipids (Packer et al., 2016). Therefore, humans need essential fatty acids linoleic acid (C18: 2n-6) and alpha-linolenic acid (ALA, C18: 3n-3) from food sources. Through extension and desaturation, alpha-linolenic acid can be converted to eicosapentaenoic acid (EPA C20: 5n-3) and docosahexaenoic acid (DHA C22: 6n-3) by "humans". However, this process is not very efficient because only small amount of EPA and DHA that can be synthesized (Swanson, Block, & Mousa, 2012).

Omega 3 fatty acids (EPA and DHA) produce a variety of anti-inflammatory effects and can potentially be added to food as a functional ingredient. Currently, most EPA and DHA consumed are sourced from fish oil or krill oil (Packer et al., 2016). This source is undesirable due to the fishy smell, displeasing taste, and unstable oxidation (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). This practice will diminish with the development of algae as a source of EPA and DHA (Packer et al., 2016).

The microalga *Trachydiscus* is a promising PUFAs (EPA) producer. It was often mistaken as *Chlorella* because of the similar morphologies. Some essential components in *Trachydiscus* are lipids, pigments, carotenoids, and β-1,3-glucan (Friedl, Rybalka, & Kryvenda, 2013). What distinguishes *Trachydiscus* from *Chlorella* is solid taxonomic composition and absence of chlorophyll *b* and chlorophyll *c*. The taxonomic genus of *Trachydiscus* is *Trachydiscus*. Hegewald et al. (2007) transferred a genus of algae from Xanthophyceae into Eustigmatophyceae, following detailed investigation of its characteristic (Lukavský, 2012).

Xanthophyceae and Eustigmatophyceae, are similar in phenotype and connected by their taxonomic history (Friedl et al., 2013). Their members are equally short of fucoxanthin, and because of chlorophyll *b* is also absent, they have a greenish-yellow colour. Xanthophyceae has similarities with green algae on growth. On the contrary, Eustigmatophyceae does not even have chlorophyll *c* (the only exceptions known in the algae heterokont), and their chloroplasts do not contain a hairy flagellum, which is the most distinctive characteristics of stramenopile algae.

Lukavský (2012) investigated the photosynthetic pigments in *T. minutus*. It has violaxanthin (16.2%), vaucheriaxanthin ester (7.4%), β-carotene (2.8%), zeaxanthin (0.9%), and neoxanthin (1.3%). 71% of all the pigments are chlorophyll *a* and this confirmed that *Trachydiscus* is in a taxonomy position of Eustigmatophyceae.

A novel strain, *Trachydiscus* sp. LCR-Awa9/2 or *Trachydiscus* sp. "Awarua", is found in the Awarua wetland in the South Island of New Zealand (Figure 1). This species has very high levels of EPA, reaching 45.2% of total lipids in the cell (Thom, 2015).

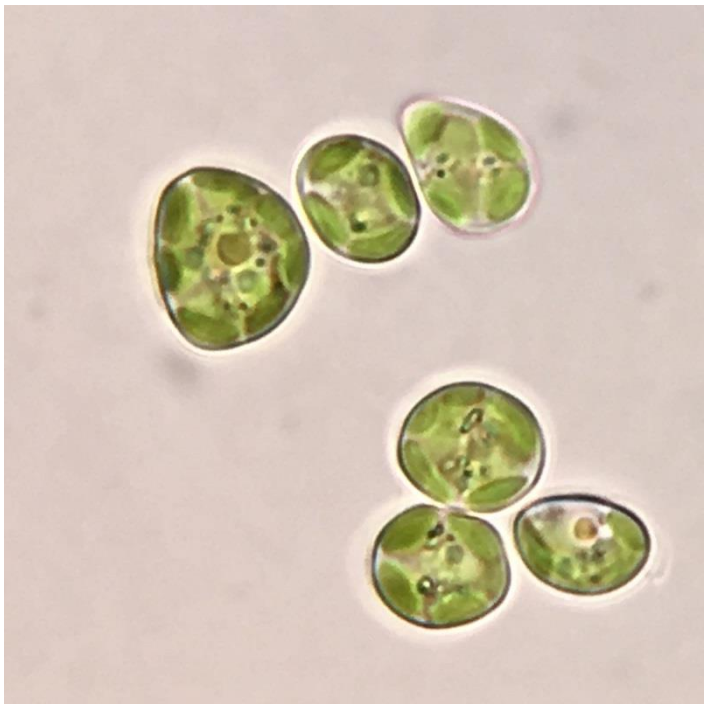

Figure 1. *Trachydiscus* sp. LCR-Awa9/2 cells.

1.2 Research Objective

The primary goal of this research is to obtain a culture medium with enhanced performance for the growth of *Trachydiscus* sp. LCR-Awa9/2. By finding the improved culture medium, high cell density culture and high EPA production are expected to be reached.

This research will be divided into three phases: the borrowing phase, one-at-a-time phase, and component swapping phase, as explained below. Culture medium development will be done in shake-flasks.

Chapter II

GENERAL LITERATURE REVIEW

2.1 Oxygenic Photosynthesis in Microalgae

Photosynthesis is a biochemical process of the plant, which converts solar energy into chemical energy which is then stored as biomass, accompanied by fixation of carbon dioxide and the formation of molecular oxygen (Posten, 2013).

Photosynthesis is effected by assimilation, under lighting, of inorganic carbon and mineral nutrients dissolved in the medium (Pruvost, Cornet, & Pilon, 2016). Cultivation of photosynthetic microorganisms requires sunlight or artificial light sources, within the photosynthetically active radiation (PAR), spectrum ranging between 400 and 700 nm. Photosynthesis also requires an inorganic carbon source, such as dissolved CO_2. Moreover, there are mineral nutrients, including major nutrients like N, S, P, and micronutrients such as Mg, Ca, Mn, Cu, and Fe. Also important are medium conditions, including pH, temperature, and dissolved oxygen.

Quantitatively in microalgae cultivation, the primary variables that affect the growth of the microalgae and productivity are light; the concentration of assorted compounds in a liquid phase such as dissolved inorganic carbon, dissolved oxygen, and growth mineral nutrient; and medium conditions such as pH, temperature, and biological contamination (Pruvost et al., 2016). The aim is normally to determine the conditions for maximum growth.

The process of photosynthesis is divided into the light and dark reactions (Richmond & Hu, 2013). As illustrated in Figure 2, in the light reaction which occurs at sites attached to the photosynthetic membrane, light energy transforms into chemical energy. This stage produces biochemical reductant $NADPH_2$ and high energy compound ATP. In the so-called dark reactions that occur in the stroma, $NADPH_2$ and ATP is used for subsequent biochemical reduction to produce carbohydrates from carbon dioxide. Contrary to the name, the dark reactions do not require darkness, but neither do they require light.

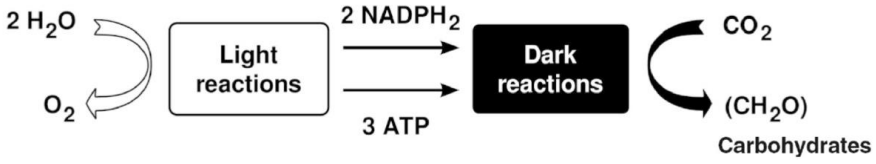

Figure 2. Main products of the light and dark reactions of photosynthesis (Richmond & Hu, 2013).

The light reaction of photosynthesis occurs in the thylakoid membrane which has five major complexes (Figure 3): light-harvesting antennae, photosystem II (PS II) and photosystem I (PS I), cytochrome b 6 / f, and ATP synthase.

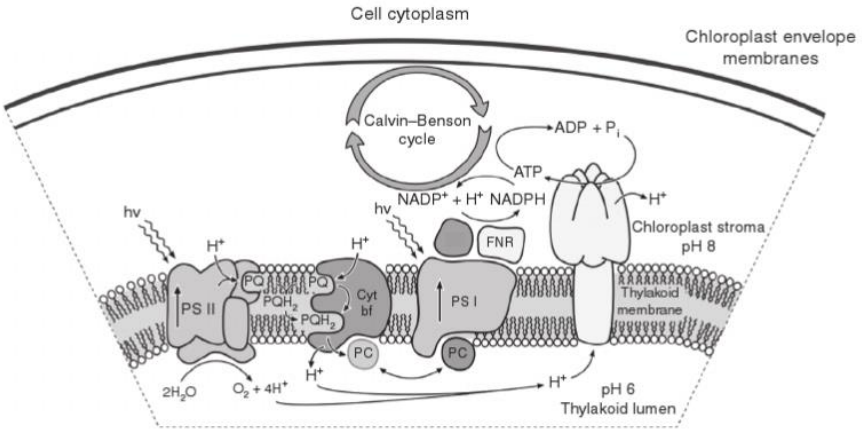

Figure 3. The vectorial arrangement of photosystems I and II, the cytochrome, b 6 /f complex (Cyt bf), and the ATP synthase within the thylakoid (Richmond & Hu, 2013).

Electrons are eliminated from H_2O molecules causing the evolution of O_2 as a by-product (Richmond & Hu, 2013). O_2 is then transported outside the thylakoid. Protons are translocated from an external space (stroma) into the intra-thylakoid space during the light-induced electron transport. The generation of ATP from ADP and inorganic phosphate occurs when protons flow through the ATP synthase to the stroma. Moreover, $NADPH_2$ is also formed on the stromal side of the thylakoid.

In the dark reaction, the fixation of carbon dioxide takes place using $NADPH_2$ and ATP which generated from the light reaction (Richmond & Hu, 2013).

Conversion reaction of CO_2 into carbohydrates (or other compounds), commonly called the Calvin-Benson cycle, occurs in four different phases as shown in Figure 4 (Richmond & Hu, 2013).

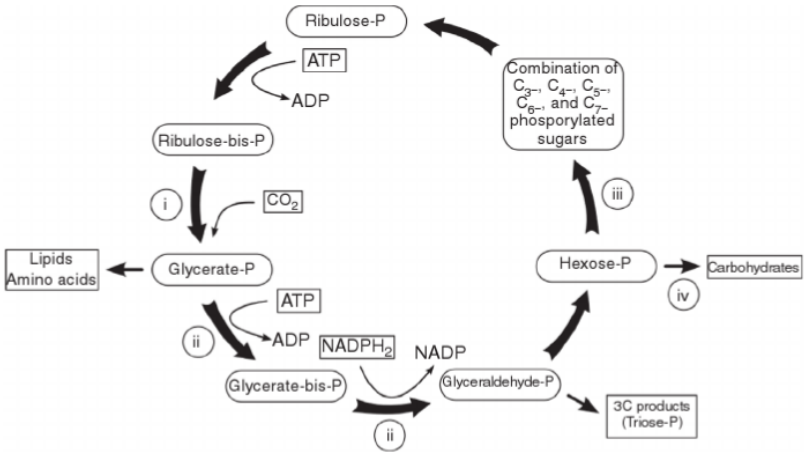

Figure 4. The photosynthetic carbon fixation pathways – the Calvin– Benson cycle (Richmond & Hu, 2013).

First is the carboxylation phase (Richmond & Hu, 2013). CO_2 is added to the 5-carbon sugar within ribulose bisphosphate (ribulose-bis-P) to form two molecules of phosphoglycerate

(glycerate-P). This reaction is catalyzed by enzyme ribulose bisphosphate carboxylase/oxygenase (called Rubisco). Second is the reduction phase. Energy must be added in the form of ATP and $NADPH_2$ in order change phosphoglycerate into 3-carbon products (triose-P). This happens in two steps. Phosphorylation of phosphoglycerate resulting in diphosphoglycerate and ADP, and the reduction of diphosphoglycerate (glycerate-bis-P) into phosphoglyceraldehyde (glyceraldehyde-P) by $NADPH_2$. Third is the regeneration phase. Ribulose phosphate (ribulose-P) is remade for further CO_2 fixation process in a complex series of reactions that combines 3-, 4-, 5-, 6-, and 7-carbon sugar phosphate. Aldolase and transketolase enzymes produce 5-carbon sugar from 6-carbon and 3-carbon sugar. The fourth is production phase. Carbohydrates are considered as the main final product of photosynthesis, but fatty acids, amino acids, and organic acids are also synthesized in the dark reactions. Several end products can be formed under different conditions. The intensity of light, CO_2 and O_2 concentration, and nutrition are factors that affect the formation of the final product.

Photorespiration is the process in which organic carbon is transformed to CO_2 without metabolic advantage (Richmond & Hu, 2013). In this process, Rubisco, serves as oxygenase, catalyzing reactions of O_2 using ribulose bisphosphate to produce phosphoglycolate. After dephosphorylation, glycolic is converted in a few steps into serine, ammonia, and CO_2. Photorespiration depends on the relative concentrations of oxygen and CO_2. The high ratio of O_2/CO_2 (O_2 concentration is higher than CO_2) triggers this process. For optimal results in the mass culture of microalgae, it is necessary to minimize the effects of photorespiration. This can be accomplished by stripping oxygen while enriching CO_2.

Microalgae accumulate lipids when the environment does not support growth, for instance, nitrogen and phosphate starvation (Bellou et al., 2014). Lipid synthesis process is illustrated in Figure 5.

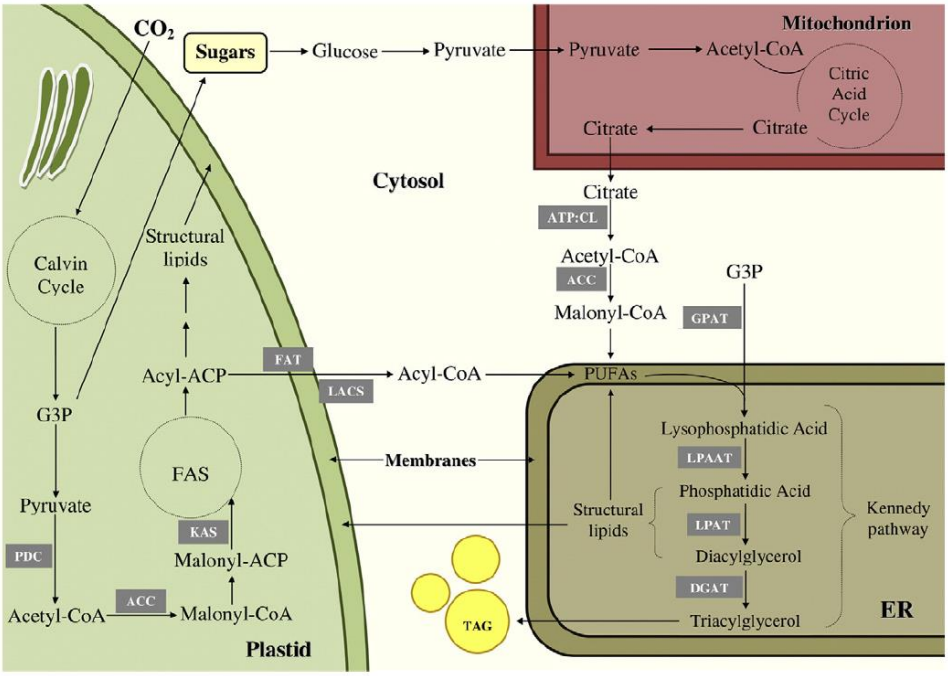

Figure 5. The scheme of lipid synthesis in microalgae (Bellou et al., 2014).

Through photosynthesis, carbon dioxide (CO_2) is transformed to glycerol-3-phosphate (G3P) as in Figure 5 (Bellou et al., 2014). It is an antecedent of some storage materials, such as polysaccharides and lipids. Through catalytic reaction by pyruvate dehydrogenase complex (PDC), G3P is converted to pyruvate and then to acetyl coenzyme A (acetyl-CoA). This phase starts the lipid biosynthesis pathway that occurs in the plastid. Acetyl-CoA can also be produced through biochemical pathways that allow transformation of polysaccharides into lipids. The pathway, during sugar assimilation, is used by heterotrophic oleaginous microalgae (microalgae that is able to accumulate lipid more than 20% of their biomass).

In detail, the G3P conversion under light limitation produces energy via glycolysis that occurs in the cytosol (Bellou et al., 2014). Afterward is the citric acid cycle taking place in the mitochondrion. Some environmental stresses, for example, nitrogen or phosphate starvation, can interfere with the citric acid cycle (by inhibiting NAD^+ -isocitrate dehydrogenase). It results in citrate accumulation in the mitochondria which later excreted in the cytosol. Cytosolic ATP-dependent citrate lyase (ATP: CL) transforms citrate into oxaloacetate and acetyl-CoA. Then, it is converted into malonyl-CoA by cytosolic acetyl-CoA carboxylase (ACC) and can be used for fatty acids elongation in the membrane of the endoplasmic reticulum (ER). In spite of biosynthesis of acetyl-CoA is considered to be important, the promising phase in fatty acid biosynthesis is the acetyl-CoA carboxylation to produce malonyl-CoA. That reaction is catalyzed by the ACCs placed in the plastid or cytosol.
The synthesis of long-chain polyunsaturated fatty acids needs particular elongases and desaturases, which act mainly on palmitic, stearic, and oleic acid (Bellou et al., 2014). Fatty acid elongation takes place in plastids and endoplasmic reticulum and needs acyl-CoA and malonyl-CoA as substrate plus one molecule of ATP and two molecules of NADPH per-unit C-2 carbon chain elongation. Desaturases are specific to the location, and number and stereochemistry of the double bonds in fatty acids.

2.2 Mass Cultivation of Microalgae

2.2.1 Sterilization

Sterilization of the culture medium must be done to avoid additional contamination. All laboratory equipment and the medium must be sterilized before use (Lee, 2016). There are some sterilization types which can be classified into four categories: sterilization using heat, filtration, electromagnetic waves, and chemical (Kawachi & Noel, 2005).

The most common sterilization method is by exposing the material (high temperature resistant material such as glassware, metallic instruments, and liquid culture media) to high temperature (usually 121 °C for 15 minutes in using an autoclave). The other method is filtration which can be applied to sterilize liquid containing heat-labile component such as vitamins. A variety of filters with different pore size, composition, and size is available. To sterilize a culture medium, the pore size of the filter needs to be less than 0.22 µm. For a small volume, sterile syringes with disposable filter units are commonly used (Kawachi & Noel, 2005). Additionally, 70% ethanol solution is also frequently used for sterilization of equipment.

2.2.2 The cultivation fundamentals

There are two types of culture media used for microalgae cultivation, namely solid media (agar) and liquid culture media. For solid media, a streaking method is commonly used, which could be done using agar plate in a petri dish or agar slant in a test tube (Bux & Chisti, 2016). The agar cultures offer some advantages such as ease of isolation from contaminants, but they also have disadvantages such as difficulty with scaling up.

A liquid culture can be used for a batch or continuous culture, batch culture being the most common. Batch culture is performed by cultivating a microalgae inoculum in a limited volume of culture medium, in a culture vessel that is incubated in a favourable growth environment (Bux & Chisti, 2016).

In contrast, continuous culture is run in a photobioreactor, where a fresh supply of culture medium is added and the culture is harvested continuously at the same flow rate. This method allows the culture to remain in the exponential phase for an indefinite period (Wood, Everroad, & Wingard, 2005).

The cultivation of microalgae can be performed in plates, shake flasks, and photobioreactors. Plates and shake flasks are preferred for preliminary experiments to find most favourable growth condition, such as culture media composition. By using plates or flasks, culture media with different compositions can be tested at the same time.

Photobioreactors are used to scale up microalgae production. There are two systems in microalgae cultivation (Pruvost et al., 2016). Currently, an open system like a natural pond and raceways is the most widely applied technology for cultivation outdoors. In fact, they have been used for decades at industrial scale. The disadvantage of this system is the high risk of biological contamination from other species of microalgae, bacteria, and predators due to direct contact of growing medium with the atmosphere. Therefore, only resistant species can be cultured for long periods of time. Direct contact between the growing medium and the atmosphere also makes control difficult. For example, it is difficult to maintain optimum temperature. Another disadvantage is that the concentration of CO_2 in the atmosphere is relatively low. This will result in a small concentration of dissolved carbon in the culture medium, which is often inadequate to meet the needs of photosynthetic microorganisms for intensive production of biomass. Microporous gas diffusers can be used to provide CO_2 (in the form of fine gas bubbles) for raceway ponds (Chisti, 2016).

Closed systems, often called "photobioreactors" (PBRs), reduce risks of external contamination and provides better control for growing conditions. For example, CO_2 can be sparged into PBRs (Pruvost et al., 2016).

Various types of photobioreactors have been designed and developed for the production of microalgae (Singh & Sharma, 2012). The airlift photobioreactor is a reactor with two interconnected zones. One tube called a riser has a gas inlet, while the other called downcomer

which does not receive gas. Mixing is done by bubbling gas through the sparger into the riser tube without physical agitation. The riser is similar to a bubble column in which gas is sparged move up randomly. This reduces the density of the riser to make the liquid move upwards. This upward movement is assisted by the gas rising from the riser. Airlift reactors have the advantage of creating a circular mixing pattern where a liquid culture is passing continually through phases of darkness and light that give the effect of flashing light for the algae cells. The residence time of gas in various zones affects the performance of several parameters such as gas-liquid mass transfer, heat transfer, mixing and turbulence.

Illumination, agitation, temperature, nutrition, and pH are some of the parameters that affect the cultivation process.

Illumination and temperature

One of the primary physical factors controlling the growth and metabolism of microalgae is light. It is an energy source for photosynthesis. Under high irradiance, photoacclimation can occur (Gigova, Ivanova, Gacheva, Andreeva, & Furnadzhieva, 2012), where some changes occur at optical, biophysical, biochemical, ultrastructural, physiological, and molecular levels (Vonshak & Torzillo, 2013). The other significant factor is temperature which is mainly related to some changes in cellular structural components (particularly lipids and proteins) (Gigova et al., 2012), the rates of cell reactions, the nutrition needs, and the biomass composition (Pirt, 1975).

As light is a primary need for photosynthesis, the amount of light will significantly affect the process (Fondriest Environmental Inc., 2014). Not all light can be used for photosynthesis, but only the visible light with a range of 400 to 700 (blue to red) is considered as active radiation for photosynthesis (Figure 6).

As explained in Chapter 1, 71% of all pigments in *Trachydiscus minutus* is chlorophyll *a*. It also has 2.8% β-carotene. Based on Figure 7, chlorophyll *a* has the best absorption rate in a range of wavelength 400-500 (cool light).

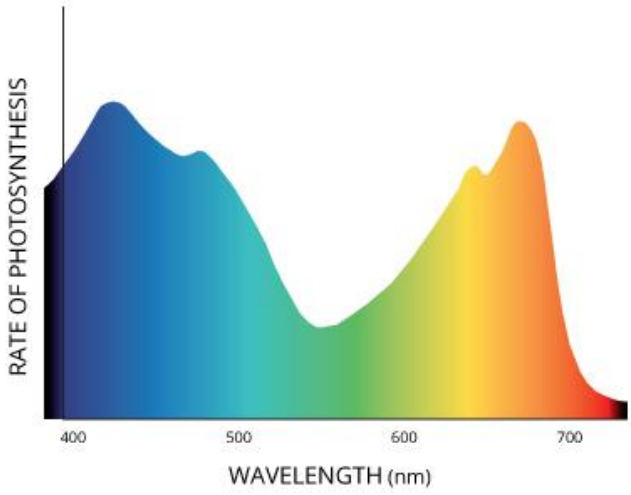

Figure 6. The rate of photosynthesis in different wavelengths (Fondriest Environmental Inc., 2014).

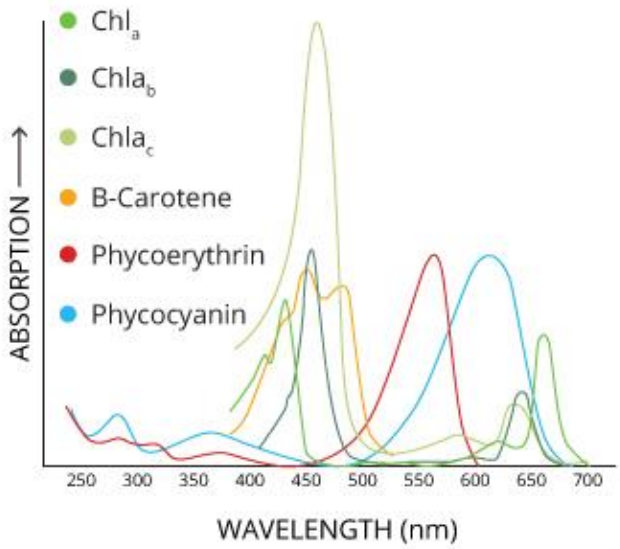

Figure 7. Pigments absorption in different wavelengths (Fondriest Environmental Inc., 2014).

Some studies of the light intensity and temperature effect on *Trachydiscus minutus* growth are provided by Gigova et al. (2012) and Cepák, Přibyl, Kohoutková, and Kaštánek (2014). Gigova et al. (2012) cultivated *T. minutus* at two levels of light intensity (reported as 132 and 2×132 µmol $m^{-2}s^{-1}$) and at six different temperatures (15, 20, 25, 32, 40, and 42 °C). The optimum condition was obtained with a light intensity of 2×132 µmol $m^{-2}s^{-1}$ at 25 °C. The optimum light intensity from Gigova et al. (2012) was lower than Cepák et al. (2014), who observed the best growth rate a light intensity of 470-1070 µmol $m^{-2}s^{-1}$ at 28 °C.

Table 1. Light intensities and temperatures in some *T. minutus* studies.

Reference	**T** (°C)	**Light intensity** (µmol $m^{-2}s^{-1}$)
Řezanka et al. (2010)	28	450
Řezanka, Lukavský, Nedbalová, and Sigler (2011)	27	160
Gigova et al. (2012)	25	246
Cepák et al. (2014)	28	470
Alexandrov, Iliev, and Petkov (2014)	26	300
Řezanka, Lukavský, Sigler, Nedbalová, and Vítová (2015)	25	80
Pádrová et al. (2015)	27	48
Gigova and Ivanova (2015)	28	132

Mixing and Turbulence

The practical aim of mixing is to avoid the cells settling at the bottom of the cultivation vessels. The settling of cells occurs when the flow is too slow and this results cell clumping and a death space, where both the death and decomposition of cells occur. This will reduce the output and poorly affect the quality of the product (Bux & Chisti, 2016). Mixing is necessary to ensure the efficiency of the light and nutrient usage for photosynthetic productivity. It also decreases the

oxygen concentration, particularly when the culture has a highly active photosynthesizing cell density which creates high concentration of dissolve oxygen (Richmond & Hu, 2013).

Some methods can be used to maintain a well-mixed culture, such as gas sparging, shake incubating, or using a stirrer vessel. Most *Trachydiscus minutus* cultivation has used a gas sparger to keep the cells well dispersed in the culture. Řezanka et al. (2010) sparged 2% CO_2 enriched air with a flow rate of 500 mL/min into 250 mL flask which contained 120 mL cultures. Alexandrov et al. (2014) used 0.5% CO_2 enriched air with a flow rate of 120 mL/min into an 8 L glass vessel in a vertical layer. In contrast, Řezanka et al. (2011) shook the flask three times a day, similar to Krienitz and Wirth (2006) who worked with a freshwater EPA producer microalga, *Nannochloropsis limnetica*.

In another study of *N. limnetica*, Parupudi et al. (2016) cultivated the microalgae in a bubble column reactor. CO_2 enriched air was sparged from the bottom of the reactor to ensure that the culture was well aerated and mixed.

Sobczuk, Camacho, Grima, and Chisti (2006) investigated the effect of mechanical agitation on the microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum* by cultivating both in shake flasks that were incubated in an orbital shaker incubator (shaker speed 200 rpm) and in a 5 L stirred bioreactor (agitation speed range 150 – 550 rpm). They found that increasing mixing in the deep photobioreactor could improve biomass production due to the rise of light regimen, but high speed of mixing along with aeration in the photobioreactor could harm the cells. It would cause smaller gas bubble and small gas bubble rupture was more harmful to the cells. In conclusion, different species of microalgae have different tolerance to mechanical stress.

2.2.3 Growth phases

There are four phases that occur in a batch culture which indicate changes in the biomass and the culture condition.

The first phase is a lag phase, where the duration of this phase can be determined by plotting the cell density logarithm with time. The duration of the lag phase is the intersection of the extrapolated straight line (exponential phase line) and the initial cell density that is plotted along the time axis as defined in Figure 8 (Pirt, 1975).

It may be a period of adaptation to new environment conditions, for instance changes in nutrient composition or culture condition (temperature, light intensity, and agitation) (Lee, 2016). The lag phase may also be caused by dying cells in the inoculum. For example, the culture in the cultivation in Figure 8 was inoculated from MLA medium, so the cells needed to adjust to the new culture medium (Svetoslav medium) in the first 2 days. This phase can be avoided or reduced by culturing an inoculum at its late exponential phase in the same culture medium and environment as the inoculum (Lee, 2016).

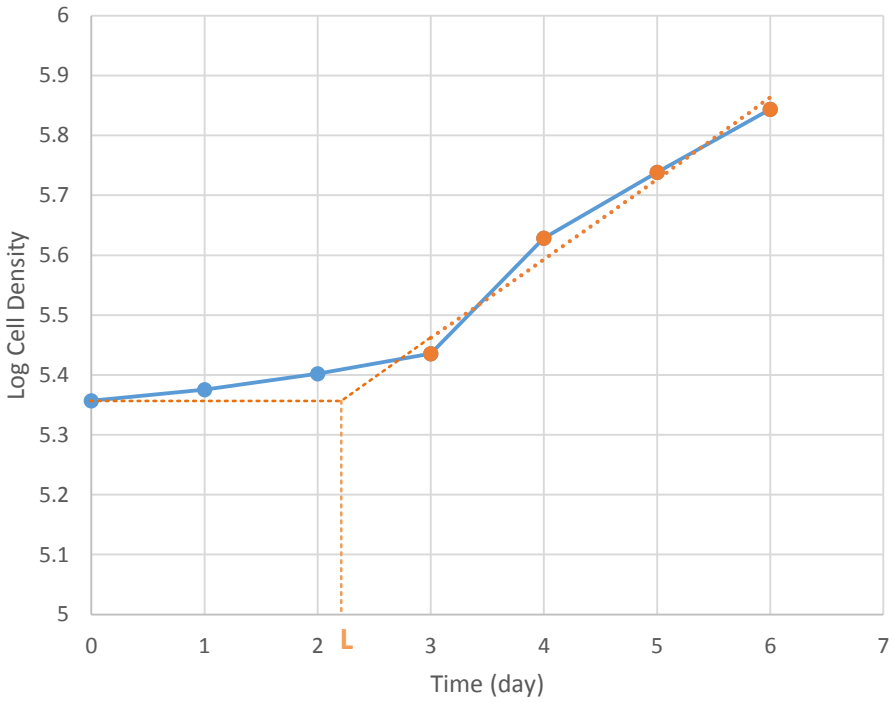

Figure 8. The lag period (L) in the growth of *T.* sp. LCR-Awa9/2 in Svetoslav medium.

The Y axis should be expressed in a log scale in order to identify the lag phase. If it is expressed in a regular scale, it will look like it has a long lag phase. The difference can be seen in Figures 9 and 10.

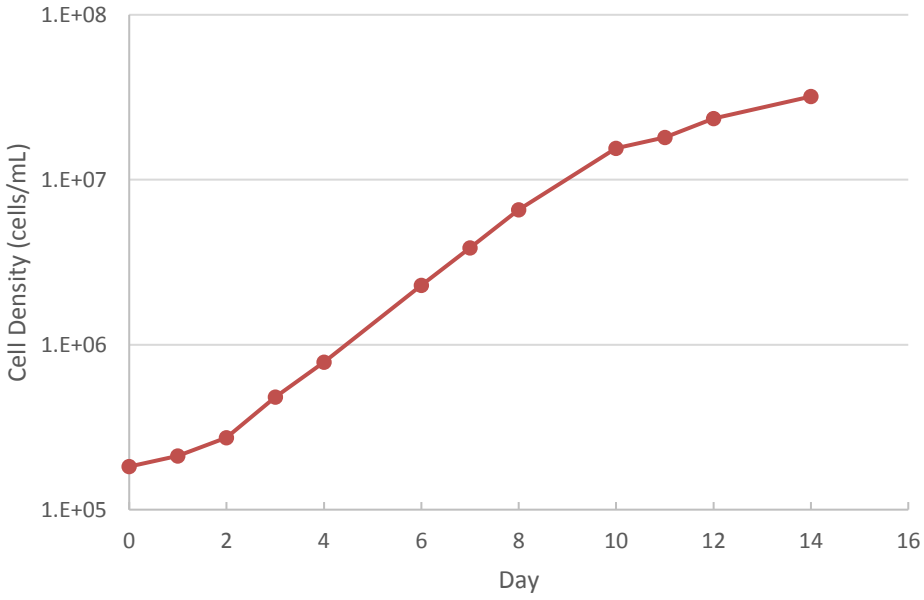

Figure 9. The growth of *T.* sp. LCR-Awa9/2 in Svetoslav medium where the cell density is plotted in a log scale.

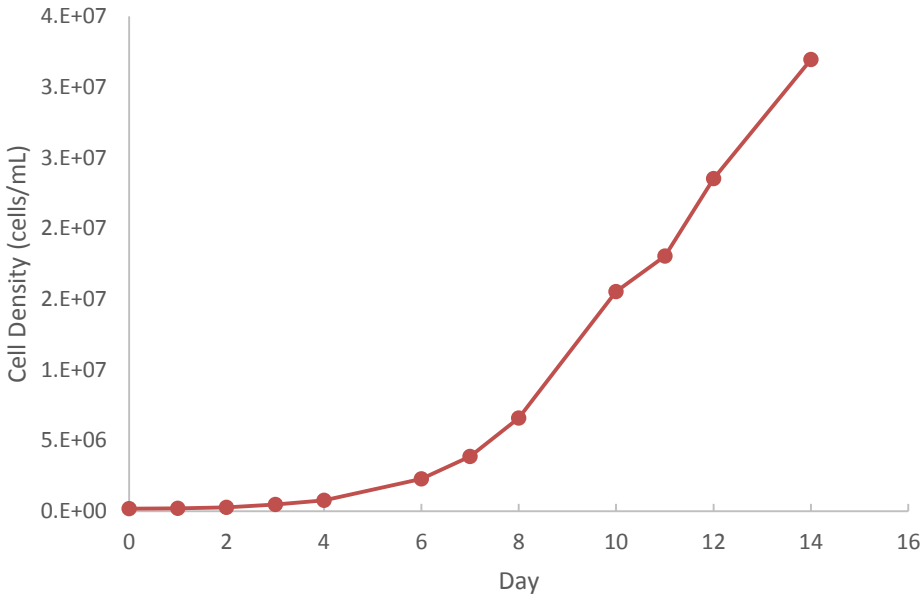

Figure 10. The growth of *T.* sp. LCR-Awa9/2 in Svetoslav medium where the cell density is plotted on a linear scale. The data are the same as Figure 9.

The second phase is an exponential phase where the cells have adjusted to the new environment and begin to grow and multiply (accelerating growth phase) and then increase to the exponential growth phase. The cells will grow in this phase as long as nutrients and light energy are available (Lee, 2016). In the exponential phase, the population growth follows this equation:

$$\frac{dN}{dt} = \mu N \quad (1)$$

$$N_t = N_0 e^{\mu t} \quad (2)$$

Solving equation 2 for μ:

$$\mu = \frac{ln^{N_t}/_{N_0}}{\Delta t} = \frac{lnN_t - lnN_0}{\Delta t} \quad (3)$$

N_0 : The population size at the beginning of the time interval
N_t : The population size at the end of the time interval
μ : The rate of increase
Δt : The length of the time interval (t_t-t_0)

N can be expressed by dry weight of the biomass (g/L) or cell density (cells/mL). μ is always expressed per unit time (t^{-1}) (Wood et al., 2005).

μ is related to doublings per day (k) by this equation:

$$k = \frac{\mu}{ln2} \quad (4)$$

Doubling time, T_2, is expressed in the same unit as μ by this equation:

$$T_2 = \frac{ln2}{\mu} \quad (5)$$

The third phase is the stationary phase where the soluble substrate in the culture medium is exhausted. In this phase, the photosynthesis still occur and storage carbon product (starch and neutral lipid) are accumulated (Lee, 2016).

The last phase is the death phase where the growth rate decelerates due to some lethal conditions such as cell starvation (Pirt, 1975).

2.2.4 Harvesting

Some processes are commonly used for harvesting or dewatering microalgae, namely centrifugation, flocculation, flotation, filtration, sedimentation, and electrophoretic separations. Centrifugation is effective and commercially used but expensive since it requires large amounts of energy, but it is good enough to be used in a small scale, particularly at an experiment scale. There are two method used for harvesting biomass at research scale: filtration and centrifugation (Andersen, 2005).

In order to measure dry weight, Řezanka et al. (2010), Řezanka et al. (2011), and Gigova et al. (2012) use a microfiber filter with a pore of 0.6 μm. They filtered the sample and then dried it at 105 °C. In order to gain biomass for further analysis, Gigova et al. (2012), Cepák et al. (2014), Alexandrov et al. (2014), and Gigova and Ivanova (2015) used centrifugation with a speed of 5000, 10000, 1000, and 5000 g, for 15, 10, 20, and 15 minutes, respectively. Řezanka et al. (2015) and Pádrová et al. (2015) also used a centrifuge to harvest the biomass.

2.3 Culture Media

Culture media are often considered on being composed of three groups of components: macronutrients, trace elements, and vitamins (Andersen, 2005). A wide variety of media culture has been developed and used for the isolation and cultivation of freshwater algae. Some of these were from the adjustment of previous recipes to suit a particular aim while some are made based on the analysis of water from native habitats. Some are formulated after a detailed study of the nutritional requirements of organisms and several others are set after ecological parameter consideration where the chemical contents in the culture media mimic the microalgae habitat.

Freshwater media divided into three categories: synthetic, enriched, and groundwater (Andersen, 2005). Synthetic or artificial media aim to provide solutions that are simple, and their components are traceable, for experiments and routine strain handling. Typical examples are the Basal Bold medium (BBM), Blue Green–11 (BG11) medium, Zehnder medium (Z), and MLA medium. They can be made either in the form of liquid and agar. Although their components are known, distilled and deionized water can have traces of contaminants, and even ultra-pure chemicals have a nanogram or picograms amount of other elements.

Enriched media are created by adding nutrients to water from lakes or rivers or by enriching the synthetic medium with soil extracts, plant extracts (e.g., peat), and yeast extract (Andersen, 2005). Components of enriched media cannot be identified with certainty because the water

from lakes and rivers has a wide variety of inorganic and organic molecules. The chemical composition of the extract is also unknown. In general, enriched media are not used for physiological experiments, but the algae often develop with normal morphology in these media. The water to prepare enriched media must be relatively clean and pollution free. Water containing a large amount of humic compounds or other organic molecules can cause disruption to the study of molecular biology, especially when the nucleic acids extracted from cells are not rinsed. Some examples are Medium-Gro Algae from the lake water (Carolina Biological Supply Co., North Carolina, United States), Audouinella media, and Diatom media.

Soil water media are made by adding dry sieved garden soil to water (the soil will settle to 1-2 cm in the bottom of test tube or bottle) (Andersen, 2005). They mimic a lake or pond. Compositions of the culture media are determined by the soil (e.g., pH, conductivity, nutrients, organic buffers, vitamins), and therefore it is important to find a good and proper soil. Algae, grown in soil water media, usually have normal morphology.

A sudden osmotic pressure alteration of a culture medium will create plasmolysis stress, resulting on photosynthesis and other function inhibition. Therefore, the algal cell in a liquid medium needs to keep the balance between water intake and loss to prevent disruptive change in volume (Rai & Gaur, 2001).

The use of various culture media for *T.* sp. LCR-Awa9/2 was investigated in this project and is reported in Chapter IV.

2.3.1 Nutrient supply

Primary considerations in developing a nutrition recipe for microalgae cultivation and maintenance are:

1. Sources of nitrogen. Nitrates, ammonia, and urea are commonly exploited as the nitrogen source. The use of nitrogen sources relies on the ability of microalgae to use the source and the pH of the medium (Lee, 2016). The pH of the cultivation medium containing nitrate is likely to increase due to the elimination of a proton (H^+), while the medium containing ammonium tends to decrease because of the accumulation of H^+. The pH of media remains constant in urea-contained media. In this case, the algae should be able to produce urease to process urea. Algae contain 7-9% nitrogen by weight of the dry matter, so a minimum of 500-600 mg/L KNO_3 is needed to produce 1 g of dry cells in 1 L of the medium;
2. Minerals. These include potassium, magnesium, sodium, calcium, sulphate, and phosphate;
3. Trace elements. These are thought to include aluminium, boron, manganese, zinc, copper, iron, cobalt and molybdenum. For the solubility of the mixture items, chelating agents such as citrate and EDTA are thought to be necessary;
4. Vitamin. Some algae (e.g. Euglena and Ochromonas) requires vitamins such as thiamine and cobalamin;

5. Total salt concentration. It depends on the origin ecological of algae, for example, green algae *Dunaliella* only survive to grow in a medium containing a minimum of 0.5 M NaCl and salinity levels are ideal with 2 M NaCl;
6. pH. Most of the media for algae cultivation have a neutral or slightly acidic pH in order to avoid precipitation of calcium, magnesium, and trace elements;
7. Carbon source (CO_2/HCO_3^-) (Grobbelaar, 2005).

2.3.2 The role of major and trace elements in microalgal growth

Nitrogen (N)

Nitrogen is known as the most essential nutrient required by microalgae. Nitrogen is present in the most important macromolecules such as proteins, nucleic acids, and polysaccharides. On average, proteins and nucleic acids contain between 15% and 13% N (Shehawy & Diethelm, 2001). Microalgae have various responses to nitrogen starvation. The biomass production will be reduced significantly due to a lower regulation of photosynthesis. In order to survive, the cells will only maintain essential protein synthesis and their electron transport chain will change into a cyclic regime. They will also accumulate carbon and energy storage compounds like polysaccharides, starch, and triacylglycerols (Gigova & Ivanova, 2015).

Microalgae can assimilate N from various sources (Rai, 2001). Figure 11 illustrates the scheme of nitrogen assimilation. Nitrate, ammonium, and urea are the most commonly used nitrogen source for microalgae.

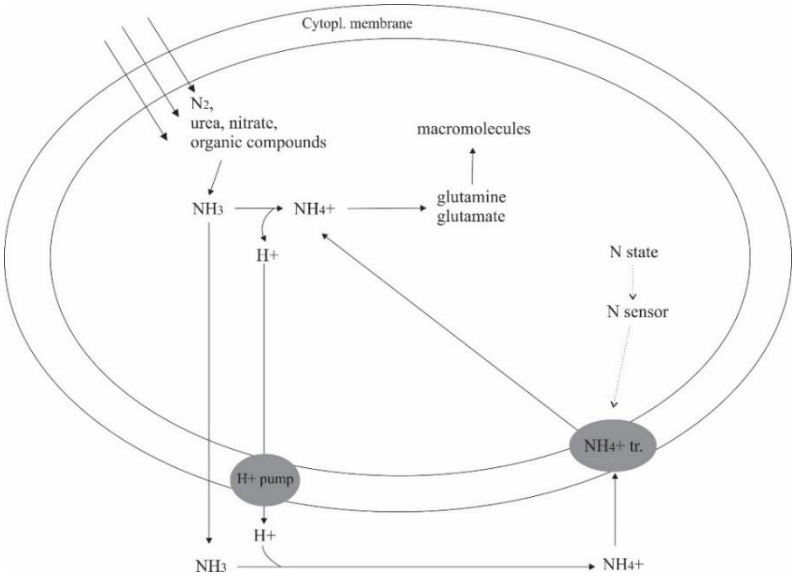

Figure 11. Schematic demonstration of the NH_3/NH_4^+ retention cycle in microorganism (Shehawy & Diethelm, 2001). ⟶ reactions including transport. ⇢ regulatory interaction. NH_4^+ tr: ammonium transport system.

Culture medium with urea as its nitrogen source is normally supplemented by nickel for the enzyme urea carboxylase and allophanate lyase that catalyse the urea amidolyase pathway (Shehawy & Diethelm, 2001).

Cepák et al. (2014) investigated the effect of different sources of nitrogen. Corresponding to the result of this experiment, *T. minutus* that was grown in urea culture produced the highest biomass, lipid, and EPA. In terms of dry biomass, the urea culture performed better than the nitrate culture. However, the urea culture produced less lipid and EPA than the nitrate culture. Chen et al. (2011) reported that *Dunaliella tertiolecta* preferred nitrate to ammonium. Although nitrate needs to be reduced to ammonium intracellularly for nitrogen uptake, a high concentration of ammonium inhibits the cell growth.

Alkhamis and Qin (2015) compared the response of *Isochrysis galbana* to different sources of nitrogen which were nitrate as $NaNO_3$, ammonium as $(NH_4)_2SO_4$, and urea (CH_4N_2O). The maximal value of biomass production was achieved when the alga was grown in a culture medium with urea as the nitrogen source, followed by nitrate. Ammonium was less effective than urea and nitrate.

Phosphorous (P)

Phosphorous is one of the fundamental building blocks which contain nucleic acid, phospholipids, and complex carbohydrates (Rai, 2001).

Sulphur *(S)*

Sulphur is an important macronutrient for microalgae cultivation. It is one component of cell composition in terms of major ionic compounds, with a cell composition range of 1.5 - 16 µg/mg dry weight (Grobbelaar, 2005). It is present in membrane sulfolipids, cell walls, vitamins, and cofactors such as thiamine, biotin, and coenzyme A. It is also needed for the biosynthesis of reduced glutathione (GHS) and cysteine (Cys) (Carfagna, Salbitani, Vona, & Esposito, 2011; Mera, Torres, Abalde, & Schroda, 2016). Mera et al. (2016) investigated the effect of sodium sulphate on the growth of freshwater microalga *Chlamydomonas moewusii* and found that the lowest concentration of sodium sulphate needed to achieve optimal growth was 0.0001 M. A toxic effect occurred at a concentration higher than 0.003 M.

Potassium (K), Calcium (Ca), Chlorine (Cl), and Sodium (Na)

Potassium, calcium, chlorine and sodium are the major ionic components of the cells. The potassium cell composition range was 1-75 µg/mg dry cells, calcium 0-80 µg/mg dry cells, and sodium 0.4-47 µg/mg dry cells (Grobbelaar, 2005). Calcium presents in some photosynthetic apparatus such as cell membrane and cytoskeleton (only in some algae) formation, membrane stability maintenance, and Photosystem II photochemical efficiency enhancement (Huang et al., 2014).

Iron (Fe) and chelating agents (EDTA and citrate)

Chelated iron is commonly used for microalgal culture media because iron solubility is often restrained in aerated or oxygenated conditions. Chelated iron is helpful in order to facilitate iron uptake, as ferric or ferrous salt have a very low solubility with some anions which will limit the iron availability (Rizwan, Mujtaba, & Lee, 2017).

Iron plays a role in enzymatic reactions and the transport systems of microalgae. Chelated iron Fe-Na-EDTA was used in the culture medium to increase the iron availability for the microalga as iron solubility is often inadequate in aerated conditions (Rizwan et al., 2017).

Iron is known as a vital component in microalgae metabolism. It presents in cytochrome c and cytochrome b_6f, which is an enzyme that is located in the thylakoid membrane in chloroplast. The membrane mediates electron transfer from Photosystem II to Photosystem I (Concas, Steriti, Pisu, & Cao, 2014) (Schoffman, Lis, Shaked, & Keren, 2016) (Richmond & Hu, 2013). Iron is also involved in redox reactions, oxygen carrier proteins, nitrogen consumption and chlorophyll synthesis (Rizwan et al., 2017). Iron in the form of free ion, or complexes with inorganic or organic ligands, or solutions can present in two oxidation states, namely Fe (III) and Fe (II) (Ilbert & Bonnefoy, 2013). The iron complexation, oxidation, chelation, and dissociation, along with iron uptake of algae are described in Figure 12.

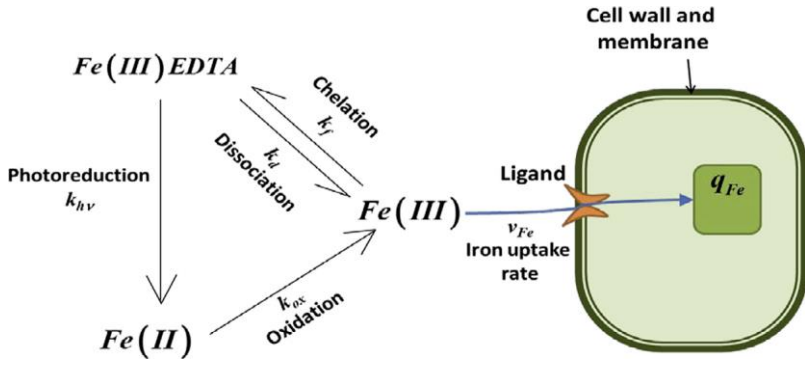

Figure 12. Schematic illustration of Fe complexation, oxidation, chelation and dissociation, and alga uptake phenomena in solution (Concas et al., 2014).

Pádrová et al. (2015) reported that a trace concentration of iron nanoparticles could increase *T. minutus* production from 10.4 to 13.7 g/L, together with a lipid content increase from 26.2% to 35%.

Magnesium (Mg)

As described in Figure 13, magnesium is the central atom of the chlorophyll molecule in the photosynthetic mechanism (Huang et al., 2014).

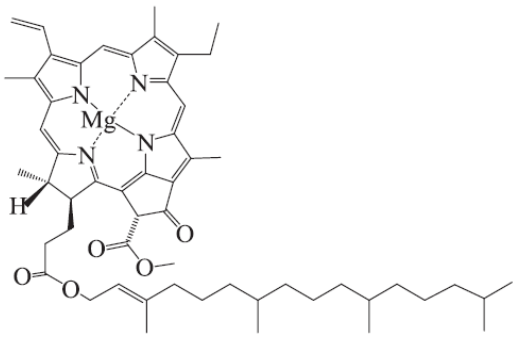

Figure 13. Structure of chlorophyll a (D'Alessandro & Antoniosi Filho, 2016). *Trachydiscus minutus* contains 71.4% chlorophyll a, along with other photosynthetic pigments such as violaxanthin (16.2%), vaucheriaxanthin ester (7.4%), β-carotene (2.8%), zeaxanthin (0.9%), and neoxanthin 1.3%) (Lukavský, 2012).

Zinc (Zn) and copper (Cu)

Zinc is an essential micronutrient for all living organisms due to its important role as a co-factor in enzymes (Huang et al., 2014). Under CO_2 limitation, the need for zinc increases. It plays an important role in carbonic anhydrase (Sunda, Proce, & Morel, 2005). It is an enzyme, which contains zinc, that catalyses the reversible reaction between CO_2 hydration and HCO_3^- dehydration in both directions (McDowall, 2004).

$$H_2O + CO_2 \leftrightarrow H^+ + HCO_3$$

CO_2 can easily diffuse in and out of the cell, therefore the conversion of HCO_3^- to CO_2 facilitates the carbon transport into the cell as HCO_3^- is poorly soluble in lipid membranes. The conversion of CO_2 to HCO_3^- facilitates carbon storage into the cell (McDowall, 2004).

Copper is essential for cytochrome oxidase which is an essential protein in electron transport between photosystem II and I (Figure 3) (Sunda et al., 2005). Copper also functions in plastocyanin in photosynthesis (Cu-binding protein), which operates in thylakoid lumen to transfer electrons between the cytochrome and PS I (Masojidek, Koblizek, & Torzillo, 2013).

The absence of zinc and cooper can result in poor alga growth and a low dry mass. Like other heavy metals, high concentrations of zinc and copper have an effect on plasma membranes which, will result in increased permeability and electrolyte leakage. This will also damage the intracellular tissue and inhibit the cell's metabolism (Zhou, Peng, Zhang, & Ying, 2012). Zhou et al. (2012) did an experiment on the bio-sorption of zinc and copper by two freshwater green microalgae, *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. They found that a 0.5 mg/L concentration of zinc and 0.2- 0.5 mg/L concentration of copper induced on increase in algae growth. The algae growth was inhibited when the copper concentration was higher than 0.5 mg/L and limited at a concentration lower than 0.2 mg/L. The culture media with a zinc concentration higher than 2 mg/L resulted in inhibition, while lower than 0.5 mg/L resulted in growth limitation. Cao et al. (2015) reported that the growth of green alga *Cladophora* reduced when the concentration of copper was below 0.1 mg/L, while zinc concentration was below 0.25 mg/L. The alga growth started to be inhibited at a zinc and copper concentration of 1 and 0.5 mg/L. Both zinc and copper affected the alga total soluble sugar content and slightly increased its protein content. They also found that zinc stimulated the intracellular calcium release while copper increased the intracellular calcium.

Molybdenum and nickel

Molybdenum and nickel are essential for nitrogen assimilation. Molybdenum together with iron in the enzymes nitrate reductase and nitrogenase, plays a role in nitrate assimilation and nitrogen fixation. This has been confirmed by Tuit, Waterbury, and Ravizza (2004) and Glass, Wolfe-Simon, Else, and Anba (2010) through their experiments on molybdenum-nitrogen limitation in marine and freshwater cyanobacteria. Glass, Axler, Chandra, and Goldman (2012) reported that some green algae such as *Chlorella pyrendoisa*, *Scenedesmus obliquus*, *Scenedesmus acutus*, and *Chlamydomonas reinhardtii* required molybdenum for nitrate assimilation.

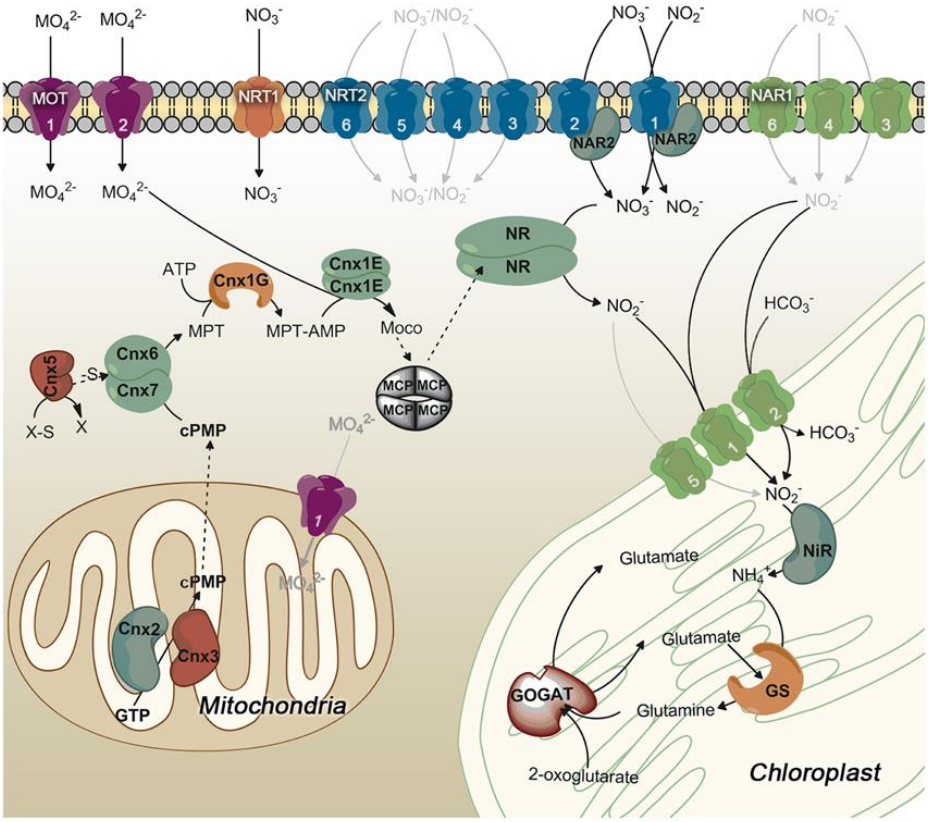

Figure 14. The model of nitrate assimilation in *Chlamydomonas (Sanz-Luque, Chamizo-Ampudia, Llamas, Galvan, & Fernandez, 2015)*.

Sanz-Luque et al. (2015) demonstrated an overview of nitrate assimilation in *Chlamydomonas* (Figure 14). Molybdenum plays roles in nitrate reductase. Molybdenum cofactor (moco) presents in the enzyme NR along with other prosthetic groups, FAD and b_{557} heme. NR is a homodimeric protein contains 100-120 kDa subunits. Its function is to reduce nitrate to nitrite with electrons from NAD(P)H.

Nickel presents in the enzyme urease, which is needed for cultures that use urea as a nitrogen source (Sunda et al., 2005). Price and Morel (1991) reported that *Thalassiosira weissflogii* needed nickel when urea was used as the nitrogen source. Dupont, Barbeau, and Palenik (2008) also confirmed that two strains of marine *Synechoccus* needed nickel when grown in urea medium.

Manganese (Mn)

Manganese is necessary as a co-factor in the water splitting enzyme in photosystem II (Richardson, Aguilar, & Nealson, 1988). It is required in higher amounts for growth under low light conditions. Its requirement for growth is much less than iron due to its involvement in fewer metabolic components. It can be sometimes replaced by iron or magnesium (Xiao-Ming, Jing-Zhong, & Pei-Yuan, 1997). However, manganese has an important function in the reactions of some enzymes, such as malic dehydrogenase and oxalosuccinic decarboxylase in the Krebs cycle (Huang et al., 2014), and superoxide dismutase, which is an enzyme that removes toxic superoxide radicals (Raven, 1990). Knauer, Jabusch, and Sigg (1999) examined

the relationship between growth rate and manganese content in the alga *Scenedesmus subspicatus*. The alga was grown in a modified OECD medium. The growth limitation occurred at a manganese concentration of less than 10^{-9} M and the inhibition at a manganese concentration of more than 10^{-5} M. The highest specific growth rate occurred in culture media with 10^{-8} M manganese concentration.

Cobalt (Co) and cadmium (Cd)

Cobalt, and occasionally cadmium, can function as an alternative enzyme for carbonic anhydrase, although their use for *Thalassiosira weissflogii* was less efficient than zinc. Cobalt is also required for vitamin B12 production (Sunda et al., 2005). Qiwei et al. (2016) found that the toxicity of cadmium for *Phacodactylum tricornutum* was around 0.07 µg per µg chlorophyll a (10 µg/L) and a lower concentration might give positive impact on chlorophyll a synthesis. Fábregas, Domínguez, Regueiro, Maseda, and Otero (2000) reported that cobalt produced lower cell density in its absence and gave higher cell density in a concentration of 0.011 mg/L.

Boron (B)

Boron was reported to be important for some identified algae species, but the specific role of boron for microalgae cultivation has not been identified (Carrano, Schellenberg, Amin, Green, & Küpper, 2009). Fábregas et al. (2000) reported that boron at 0.011 g/L was toxic because higher cell density was obtained when it was removed from the culture media. In contrast to Fábregas et al. (2000), McIlrath and Skok (1958) reported that freshwater green alga *Chlorella vulgaris* produced a higher cell number and dry biomass when supplemented by 0.0005 g/L H_3BO_2.

Vitamins

The most commonly used vitamins for microalgal culture have been biotin, thiamine, and B_{12} (Croft, Warren, & Smith, 2006). Biotin plays a role as a cofactor in some carboxylase enzymes, like acetyl coenzyme A (CoA) (Figure 5). Thiamine associates with carbohydrate and branched-chain amino acid metabolism such as pyruvate dehydrogenase, transketolase, α-ketoacid decarboxylase, and α-ketoacid oxidase. Thiamine was necessary for *H. pluvialis* growth (Fábregas et al., 2000). Biotin and B_{12} also stimulated *H. pluvialis* growth.

Chapter III

MATERIALS AND METHODS

3.1 Experimental Set-Up

The list of materials such as laboratory equipment, glassware, and chemical is provided in Appendix A. The process diagram flow of the experiment is outlined in Figure 15. All tubing that was used for the gas lines was peristaltic pump silicone tubes except the CO_2 gas cylinder to CO_2 mass flow controller line which used a stainless steel tube.

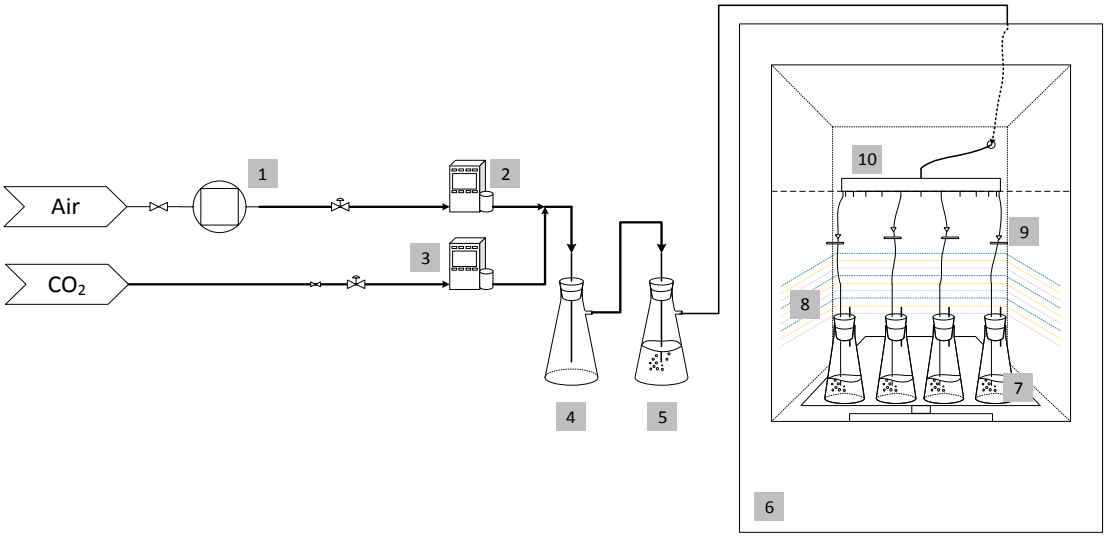

Figure 15. Experimental set up for microalgae *Trachydiscus* sp. LCR-Awa9/2 cultivation. 1. Air compressor, 2. Air mass flow controller, 3. CO_2 mass flow controller, 4. Mixing flask, 5. Humidifying flask, 6. Incubator shaker 7. Cultivation flask, 8. LED strips, 9. Gas filter 0.2 µm connected to the silicon tube by luer lock fitting, 10. Gas distributor.

The air and CO_2 passed through the mass flow controllers and were mixed in the mixing flask, resulting in 3% CO_2 enriched air. Then it entered into the humidifying flask (inner diameter of the tube from mass flow controller to humidifying flask was 4.8 mm, with 1.6 mm wall thickness). The gas flowed to the gas distributor through silicon tube (ID 6.4, wall thickness 1.6 mm) and was divided into a number channels. It flowed into the flask through 1.6 mm silicon tube (wall thickness: 1.6 mm).

Incubator

The incubator used in the experiment was a MaxQ™ 6000 Incubated/Refrigerated Stackable Shaker (Figure 16.A). The incubator had a temperature range of 10 to 60 °C and a speed range of 15 to 500 rpm. The cooler system which was provided by the incubator was useful, since the room temperature fluctuated and was often more than 26 °C while the temperature for the

experiment was 25 °C. The incubator was modified to adjust to the experimental needs, by installing LED strips, a gas inlet hole, and a gas distributor.

The fresh cell stock was kept in an OM11 Medium Orbital Shaker Incubator (Figure 16.B) (temperature control range: 28-70 °C, speed range: 40-400 rpm) because the capacity of the MaxQ 6000 incubator was only for twenty 250 mL-flasks. The fresh culture used a 500 mL flask, so the space in the MaxQ 6000 incubator was not enough. A fluorescent lamp was put above the OM11 incubator as the light source. The OM11 incubator was also used for Experiment 1 to 7 (results are written in Appendix C).

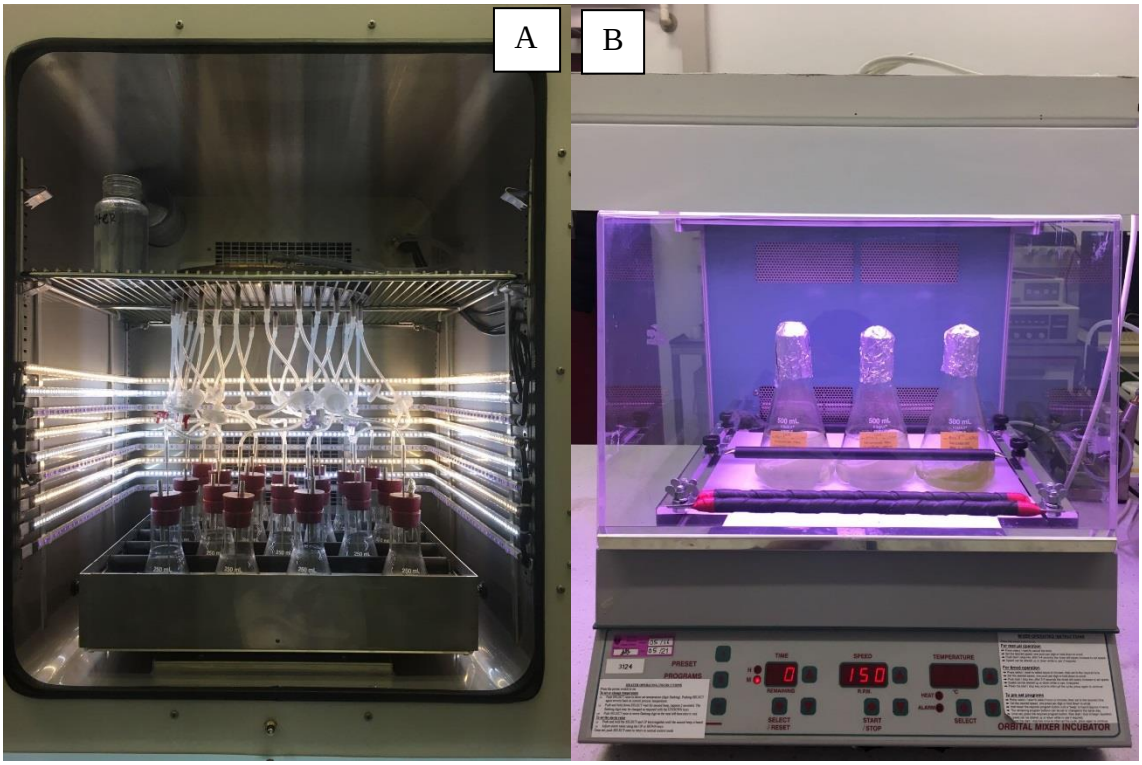

Figure 16. A. Modified MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers, B. OM11 Medium Orbital Shaker Incubator.

Cultivation Flasks

The cultivation was carried out in 250 mL and 500 mL KIMAX baffled Erlenmeyer flasks. The baffled flask was very useful to prevent the cell accumulating in the middle of the flask since the incubator shaker used orbital movement. The baffle increased the mixing efficiency by dispersing the liquid vortex.

100 mL of culture was cultivated in the 250 mL baffled flask, and the flask was sealed with a rubber stopper. The rubber stopper (size 17, top diameter: 40 mm, bottom ID 30 mm) was modified by adding two steel tube for the gas inlet (ID 1.5 mm) and vent (ID 3 mm, length: 75 mm) (Figure 17, 18). The length of the gas inlet steel tube was 200 mm and was set to be 5 mm below the culture surface.

The gas inlet tube was connected to the gas filter using silicon tube, and the gas filter was linked to the gas line from the gas distributor using a Luer lock fitting. The Luer lock fitting made the flask easy to disconnect and reconnect for sampling. The filter (0.2 µm pore, diameter 32 mm) prevented contamination entering the flask when it was brought into the laminar flow for sampling. The stopper, including the filter that was attached to it, was autoclavable.

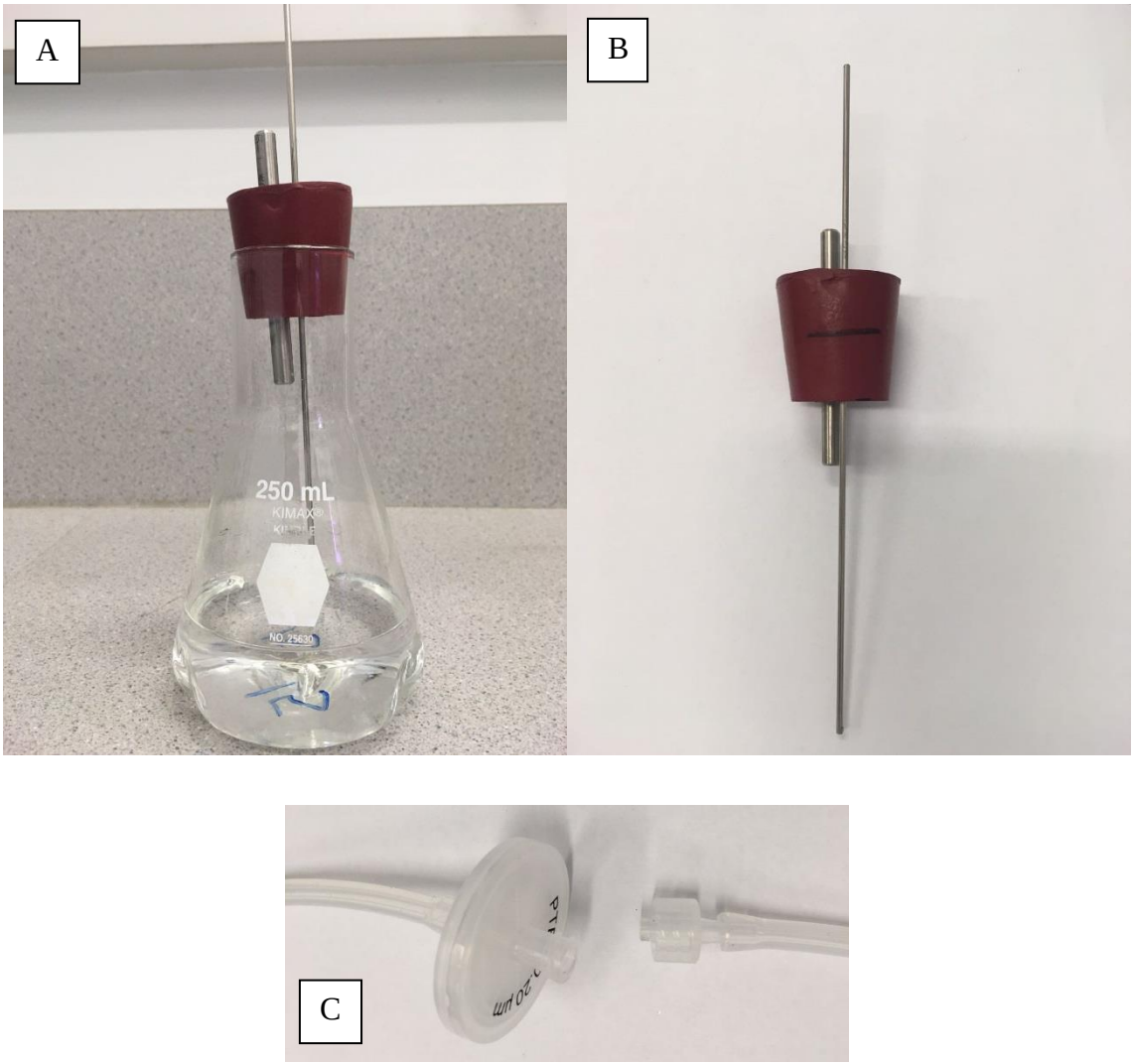

Figure 17. A. A cultivation flask, B. A rubber stopper, C. A gas filter with Luer lock fitting.

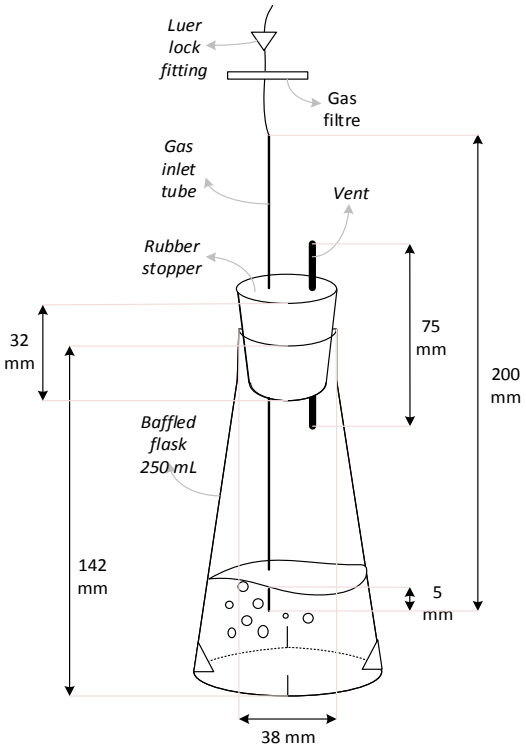

Figure 18. Cultivation flask illustration.

Gas *Supply*

3% CO_2 enriched air was sparged continuously into the flask through the tube. The compressed air and CO_2 from its gas cylinder passed through the mass flow controller into the mixing and humidifying flask. The flow rate of compressed air and CO_2 were 500 mL/min and 15 mL/min respectively. The mass flow controllers were MS Series ALICAT Scientific Mass Flow Meters (Figure 19).

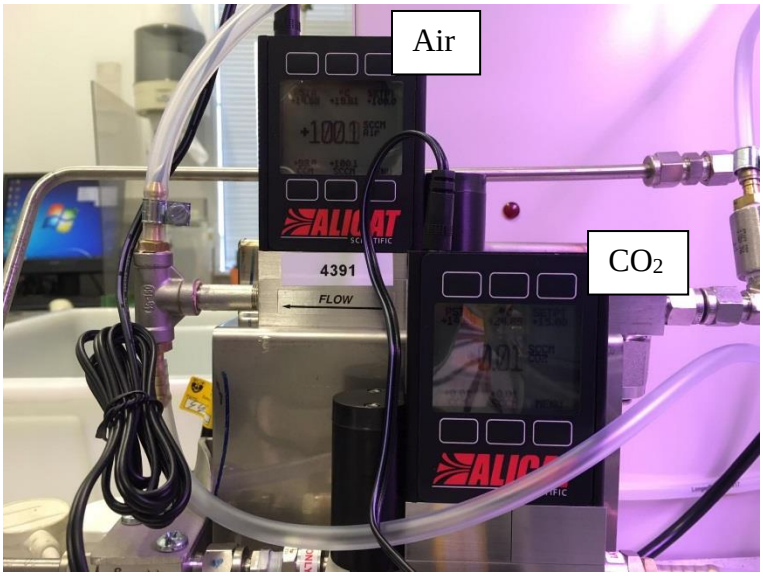

Figure 19. MS Series ALICAT Scientific Mass Flow Meter.

The gas flowed into the mixing flask and continued into the humidifying flask to be humidified. The practical aim was to prevent the gas from evaporating water in the culture flask, resulting in volume loss. Sampling would also change the volume level, but only a 20 µL sample was taken from each flask so the volume reduction was not significant and all flasks would still have the same volume. As mentioned by Gustavs, Eggert, Michalik, and Karsten (2010), microalgae photosynthesis and growth requires humid air. Fogg (2001) claimed that decreased air humidity will result in desiccation stress for freshwater algae.

After being humidified, the gas flowed to the gas distributor. The gas distributor was round with a diameter of 300 mm and 25 mm height. It was made from stainless steel sheet and had twenty-four outlets. The incubator only has space for twenty flasks, so the unused outlets were blocked by plastic stopcocks.

Temperature and Shaker Speed

The temperature was set at 25 °C and controlled by the incubator. It provided cooling and heating system to keep the set temperature.

The culture was shaken in orbital movement continuously. The purpose was to keep the culture well mixed and avoid agglomeration of cells. The shaker was run at 150 rpm. Set speed lower than 150 rpm gave poor cell distribution. The low speed was not powerful enough to break the vortex, resulted in the cells agglomerating in the middle of the vortex.

Light Source

The incubator was also modified by adding a light source. The light source used LED stripes with a combination of 3 types of light, violet, cool, and warm light. They adhered to an aluminium framework that was manufactured to fit the inner wall of the incubator. The framework could be set and detached easily.

The LED strips have three different level of light intensity. The light intensity was measured by LI-COR LI-1500 light sensor logger with LI-193 spherical submersible sensor (serial number SPQA5394). The sensor was placed in 3 different positions and the sensor was put in vertical and horizontal (lying down) position.

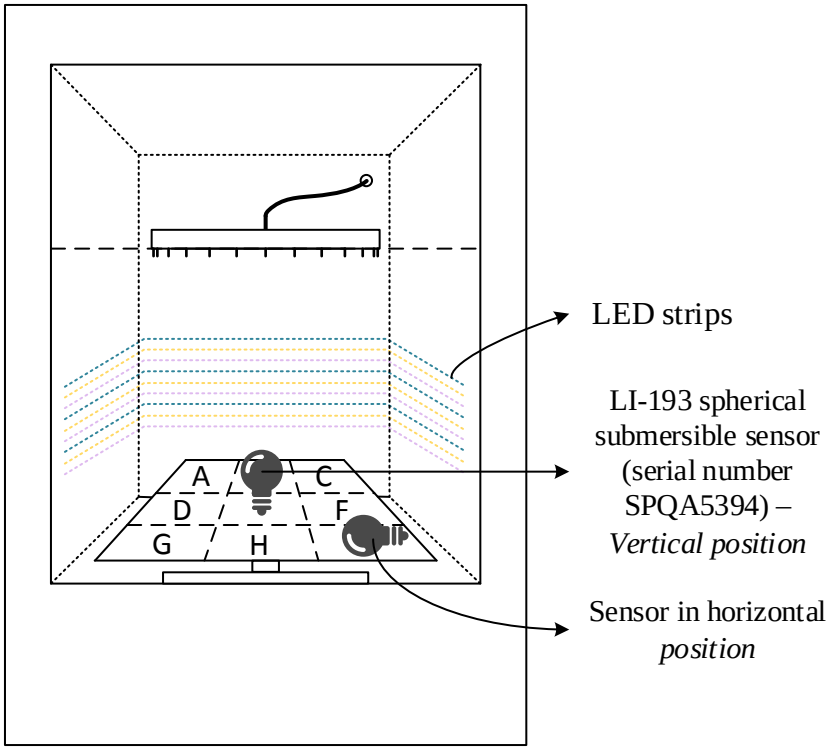

Figure 20. The illustration of the light sensor positions inside the incubator.

Table 2. The light intensity of the equipment used in the experiment.

Types of light	**Position**	**Light intensity (µmol photons/m²s)**		
		1 - Low	**2 - Med**	**3 - High**
Violet	E vertical	0.5	3	7.5
	D vertical	0.5	3.79	9.66
	A vertical	0.69	5.1	13.8
	A horizontal	0.3	2	5
	E horizontal	0.4	2.5	6.3
Cold white	E vertical	15	121	276
	D vertical	19.6	156.4	356
	A vertical	26.3	209.6	478.6
	A horizontal	11.9	95.4	217.8
	E horizontal	14.5	115.5	263.2
Warm white	E vertical	16	144	293
	D vertical	19.9	179.3	363.7
	A vertical	26.1	235.6	478.2
	A horizontal	11.9	106.5	216
	E horizontal	14.7	132.3	268.1

Types of light	Position	Light intensity (µmol photons $m^{-2}s^{-1}$)		
		1 - Low	2 - Med	3 - High
Combined Violet, Cold white, and Warm white	E vertical	31.6	267	571
	D vertical	39.8	339.3	722
	F vertical	40.2	344	732
	A vertical	52.5	449.8	958.9
	A horizontal	11.9	200	431
	E horizontal	14.7	249.2	530.6

Position had a significant effect on the light intensity. A and C (back corner, Figure 20) were the brightest positions. Therefore, in each experiment, the flask position must be randomly moved every day to avoid the uneven distribution of light. Significant differences between vertical and horizontal sensor were measured. The horizontal position was probably a better representation of the position of culture in a flask. The violet light had the lowest intensity. The intensity of the combined lights was very close to the sum of the three individual measurements. All experiments used the combined light (violet, cold, and warm), Experiment 14 – 20 used medium light intensity with a range of 200 – 250 µmol photons $m^{-2}s^{-1}$, and Experiment 21 – 23 used high light intensity with a range of 431 – 530 µmol photons $m^{-2}s^{-1}$.

For model calculations, position E horizontal was chosen as the most likely average, but clearly there is large uncertainty in the absolute light radiation.

The spectral composition of each light was measured using ASD FieldSpec 3 Spectroradiometer 350-2500 nm (Figure 21-25).

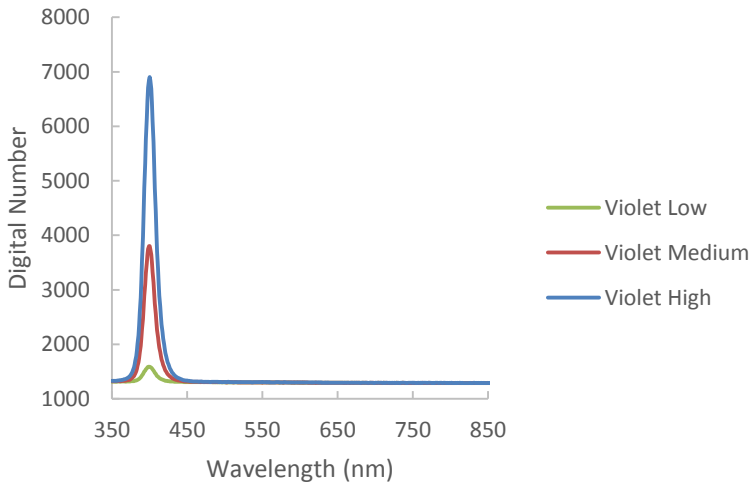

Figure 21. Violet light spectral composition.

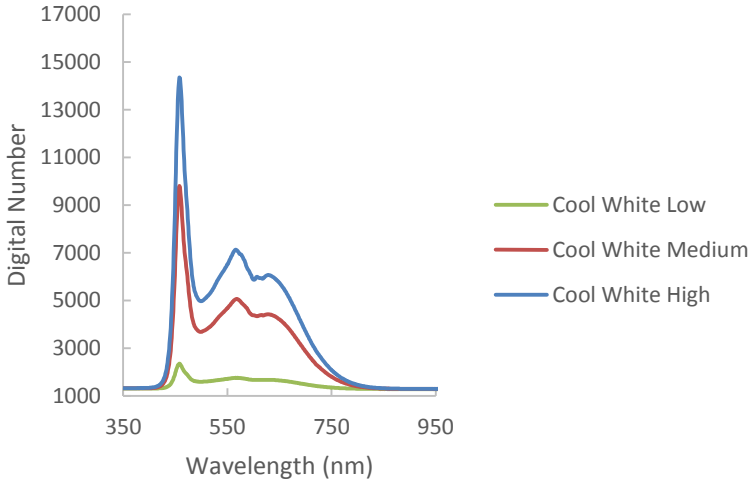

Figure 22. Cool white light spectral composition.

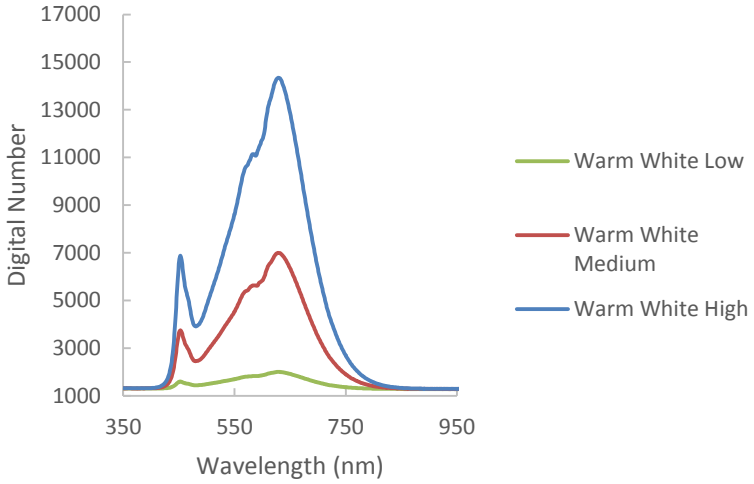

Figure 23. Warm white light spectral composition.

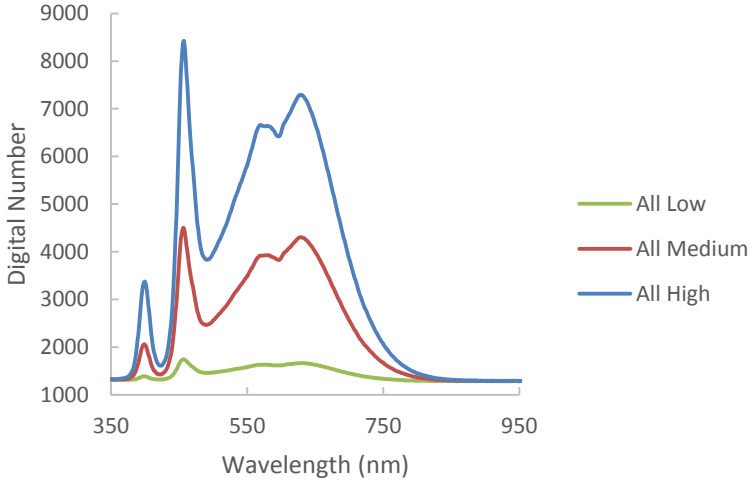

Figure 24. Combined (violet, cold, and warm) light spectral composition.

The OM11 Medium Orbital Shaker Incubator light source was a fluorescent lamp with a light intensity of 90 µmol photons $m^{-2}s^{-1}$ (vertical sensor) and 60 µmol photons $m^{-2}s^{-1}$ (horizontal sensor).

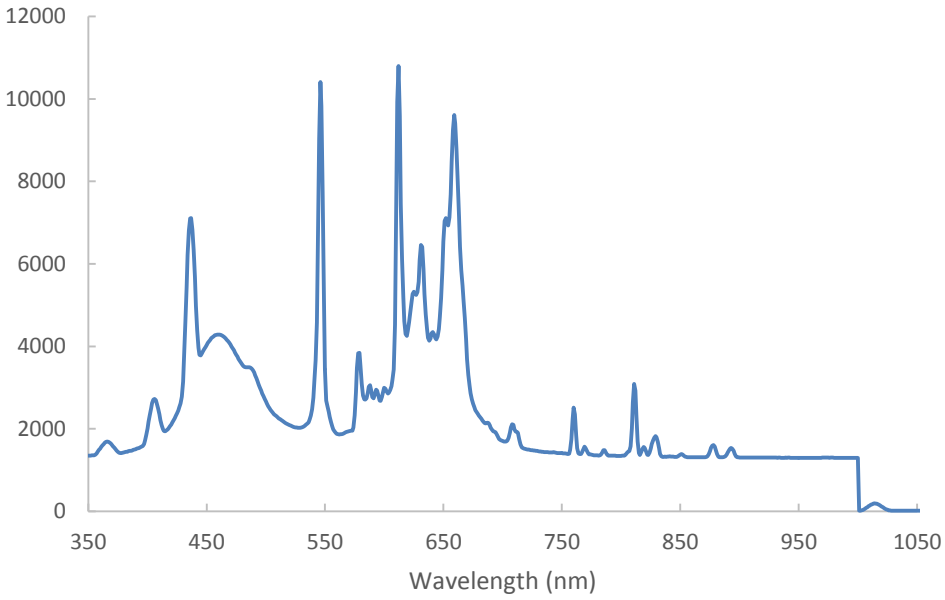

Figure 25. Fluorescent lamp wavelength.

Culture Medium

There were 8 culture media to be examined in this experiment which are Blue Green 11 Medium (BG11M) (Department Experimental Phycology and Culture Collection of Algae (EPSAG)), Bold Basal Medium (BBM) (Andersen, 2005), MLA Medium (MLAM) (Bolch & Blackburn, 1996), Modified Bourelly Medium (MBM) (Krienitz & Wirth, 2006), Optimum Haematococus Medium (OHM) (Fábregas et al., 2000), New Medium by Svetoslav (SvetoslavM) (Alexandrov et al., 2014), Zachleder and Setlik Medium (½SSM) (Zachleder & Setlík, 1982), and the Universal Zehnder Medium (ZM) (Alexandrov et al., 2014).

The method of culture media preparation used in the experiments is given in Appendix B. Bold Basal medium was prepared based on Andersen (2005) but with some modifications. Fe-EDTA (Fe concentration 1.45×10^{4} M) and $FeSO_4$ (Fe concentration 1.79×10^{5} M) were replaced by Fe-Na-EDTA (Fe concentration 1.63×10^{4} M). KOH and H_2SO_4 were neglected because the purpose of adding them was to maintain the pH at 7, but the final pH of the culture medium was 6.37 and it was still tolerable for the cultivation process.

3.2 Sterilization

There were two sterilization methods used in the experiment: autoclaving and filtration. Autoclaving is exposing the materials to heat (Kawachi & Noel, 2005). This method can be applied to materials that can resist high temperature, such as glassware, metallic instruments, and liquids. Some culture media can be sterilized using autoclave, but precipitation will occur in those that contain iron, calcium, and sodium carbonate. Culture media that contain vitamins

(MLAM, MBM, and OHM) cannot be sterilized using an autoclave because the vitamins will be destroyed by high temperature (Harrigan & McCance, 1966). Therefore, a filter can be used for sterilizing these culture media. In these experiments a sterile disposable Millex-GP syringe filter unit, pore size 0.22 µm, diameter 33 mm, was used to sterilize the culture media containing vitamins which were MLA medium, Optimum *Haematococus* Medium (OHM), and Modified Bourrelly Medium (MBM). The procedure of filter sterilization is provided in Appendix B, along with the culture media recipe.

Figure 26. A. The autoclave which was used in the experiment, B. The autoclave control panel, C. Top view of the autoclave.

Before autoclaving, pre-sterilization cleaning was required. New glassware and plastic vessels, except ready-to-use sterilized products, were cleaned before the first use. Flasks used for growing algae and reusable glass pipettes used for transferring cells were immediately rinsed with tap water after use to prevent the cell material from drying onto the glass surfaces. The glassware was washed using Down to Earth dishwashing liquid, rinsed with tap water, and rinsed again with reverse osmosis (RO) water, then dried using a clean paper towel.

The glassware to be sterilized was wrapped in aluminium foil and marked with autoclave tape. The date of sterilization was written on the tape. The same treatment was applied to some autoclavable plastic equipment such as pipette tips (stored in a pipette rack), eppendorf tubes (stored in a glass bottle), micropipettes, rubber stoppers, and reusable gas filters. For culture media that were stored in glass bottles, a free space was left in each bottle to provide space for steam and potential boiling. The bottle caps were loose to avoid an excessive accumulation of pressure, and they were wrapped in aluminium foil and labeled with autoclave tape. The materials to be sterilized were put into the autoclave basket and arranged to allow for space between individual items. The autoclave was run for 15 minutes at 121 °C.

After autoclaving, the autoclave door was not opened until the pressure was completely reduced and the temperature was less than 80 °C. The autoclave gave a sound indicator after completion. A liquid culture medium should not be left to cool to room temperature in the autoclave because it would result in precipitation. Rapid cooling outside the autoclave would minimize the formation of precipitates. Loose bottle caps were tightened after the liquid was cool. After cooling, the culture media were stored in a refrigerator while the other equipment was kept in a clean cabinet. The volume loss during autoclaving was negligible.

3.3 Starting a Culture

To start a culture, some sterile equipment was prepared in a laminar flow cabinet: baffled flasks, volumetric pipettes, a pipette bulb, volumetric cylinders and culture media. They were exposed to UV light in the laminar flow cabinet for 30 minutes to keep them sterile.

The culture medium container cap was opened for the shortest possible period to prevent contamination. The volumetric pipette was plugged with cotton wool at the wide end before sterilization. The aim was to limit the risk of contamination from the rubber bulb which may have contaminating organisms. The cotton would block the path of these organisms through the pipette.

Using a volumetric cylinder, a predetermined volume of a culture medium was poured into the baffled flask. Then the initial cell concentration of the inoculum was calculated, and a predetermined volume of starter cells was transferred into the flask using a volumetric pipette. For example, the experiment was conducted in a volume of 100 mL of culture medium (V_i) in a baffled flask. The initial cell number of the new culture medium (C_i) would be 2×10^5 cells/mL, and the cell density of the starter culture was known as C_f. The volume of starter cells culture (V_f) to be transferred into the new culture medium was calculated using this equation:

$$C_f V_f = C_i V_{i+f} \quad (6)$$

The glassware used for this work was rinsed immediately after use to prevent the cell drying and adhering to the glass.

3.4 Sampling for Cell Counting

The sampling was done one flask at a time to avoid the cells settling to the bottom of the flask. A micropipette, a rack of pipette tips, and Eppendorf tubes that had been autoclaved were prepared in the laminar flow cabinet, along with a glass beaker to put used pipette tips in. They were exposed to the UV light in the laminar flow cabinet for 30 minutes. Then the culture flask was taken from the incubator and brought to the laminar flow. An orbital movement was applied by hand to the flask for about 60 seconds to homogenize the culture, and then a volume of sample was taken from it using a micropipette. When the cell density was below 1×10^6 cells/mL, a 200 µL micropipette was used to collect 200 µL sample from the culture. After the cell density had reached that number, the sampling used a 20 µL micropipette to take 20 µL sample which was diluted with 180 µL sterile distilled water, resulting in a dilution factor of 10. The dilution was necessary to enable cell counting. The culture was returned to the incubator immediately after sampling to prevent cells sedimentation.

3.5 Cell Counting

Counting cells in environmental algae has two principal purposes. The first is to calculate the cultured population size which can be expressed as the total number of cells per unit volume of culture. The second is to evaluate the growth rate of the culture which is equal to the rate of population increase (Guillard & Sieracki, 2005). A stereo light microscope Olympus BX60 with 20× and 50× objective lenses was used for cell counting. Two plastic squeeze bottles of 80% ethanol and distilled water, a hemacytometer, a 20×25 mm coverslip, 20-200 µL and 1000 µL micropipettes, two racks of 200 µL and 1000 µL pipette tips, a box of Kimtech Wipe Laboratory Purpose tissues, and a used pipette tips container were prepared in the laminar flow cabinet. The distilled water was applied to flush the sample from the counting chambers and coverslip while the 70% ethanol was used for the final cleaning. The ethanol was also used to attach the coverslip on top of the counting slides. The 20-200 µL micropipette was used to fill the counting chamber while the 1000 µL micropipette was used to homogenize the sample that was stored in the Eppendorf tube.

The hemacytometer used in this experiment was one with a 0.1 mm deep chamber (Figure 27). It has two chambers, and each chamber has nine 1-mm squares. The chamber has various layers of division which are 250-µm squares, 250×200-µm rectangles, 200-µm squares, and 50 µm squares. The volume of a 1-mm square is 0.0001 mL.

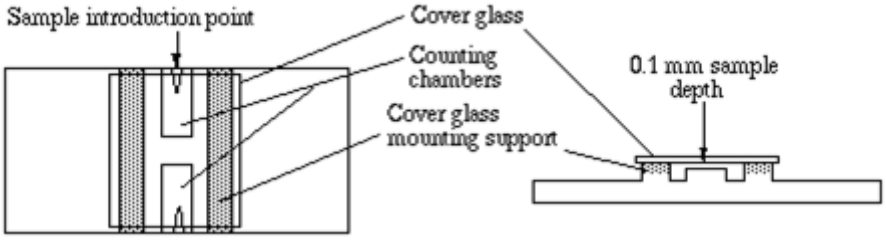

Figure 27. Parts of the Hemacytometer (The iGEM Concordia 2014 Team, 2014)

After cleaning the hemacytometer using distilled water and ethanol, the coverslip was attached to the hemacytometer using ethanol to wet the cover glass mounting support. Then, as illustrated in Figure 28, the sample in the Eppendorf tube was pulled into the 1000 µL micropipette and pushed back into the tube without producing any bubbles. This homogenization process was repeated sixty times. After that, 10 µL sample was collected using the 2-20 µL pipette to be injected into the counting chamber. The pipette tip was placed next to the entry slit of the hemacytometer, then the liquid was released, and the pipette was removed simultaneously from contact as the liquid flowed quickly and evenly into the chamber. There was no overflow of the liquid into the canals surrounding the chamber because the amount of liquid that was injected to the hemacytometer matched the chamber capacity.

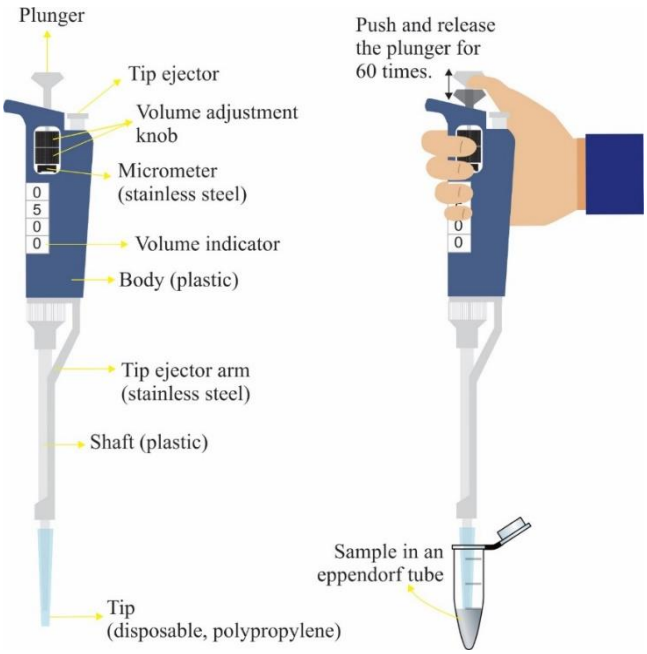

Figure 28. Parts of the micropipette and homogenize sample using a micropipette.

The hemacytometer was placed in the microscope for 30 seconds to 2 minutes before counting to allow the cells to settle. The cells in four 250-µm squares were counted. Cells in the triple lines were counted based on Figure 29. A tally device (Tally iPhone App) was used to count the cells to prevent human error especially when the counting area had many cells. The computed average number of cells per 1 mm square then multiplied by 10^4 to get a cell density which is expressed as cells/mL. The maximum number of cells counted in a 1 mm^2 square was 100.

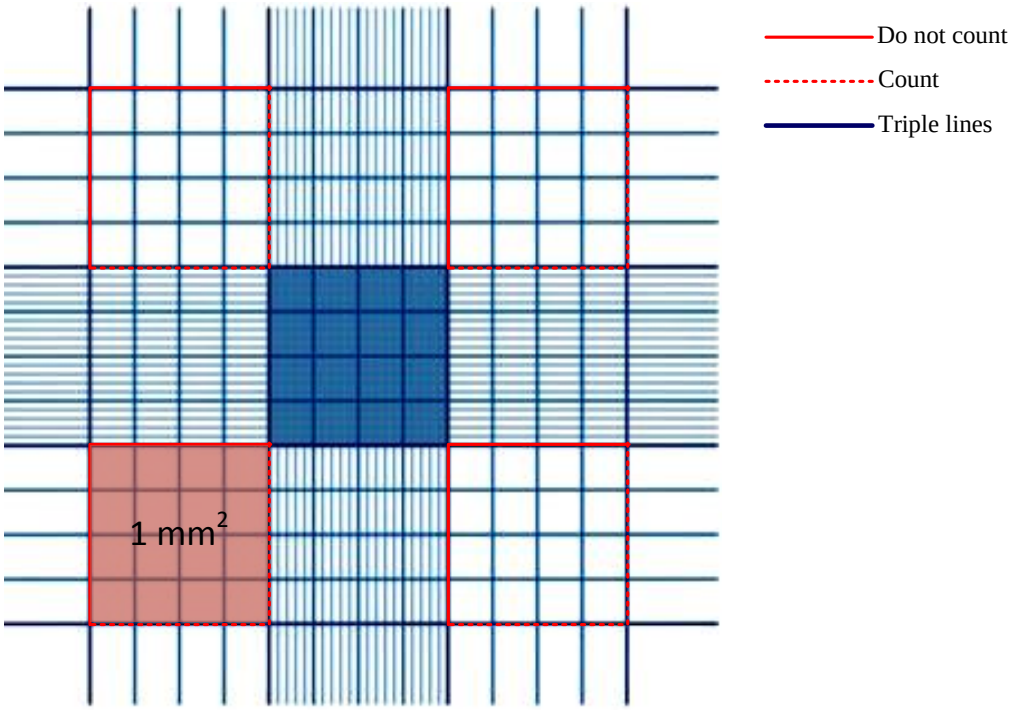

Figure 29. The hemacytometer counting chamber.

Repetitions was done when the deviation between duplicate flasks was more than 15% due to some errors during sampling.

The cells' picture was taken by iPhone SE that was connected to the microscope using iDu Lab adaptor.

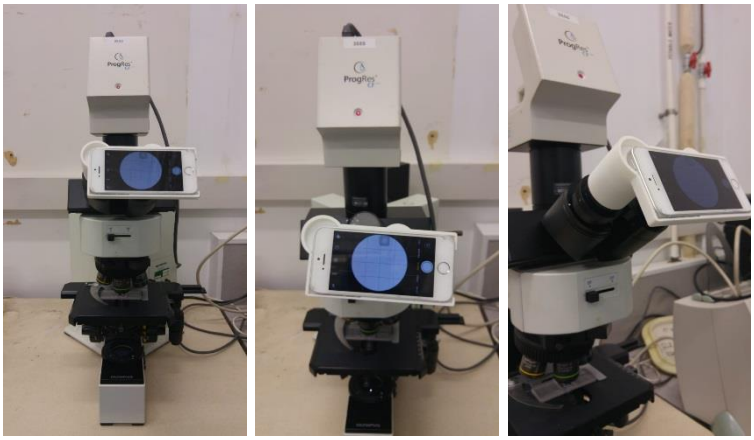

Figure 30. IDU Labcam Microscope Adaptor.

3.6 Continuous Maintenance of Microalgal Culture

The aim of the continuous maintenance for the microalgae was to keep a fresh and healthy cell population. The most common way to conduct this is by culturing the algae under controlled environmental conditions, where the strain used as the starter is at the end of its exponential growth phase (Lorenz, Friedl, & Day, 2005).

A 500 mL baffled flask, a volumetric pipette, a pipette bulb (plugged with cotton at the wide end), a volumetric cylinder and a culture medium were autoclaved and placed into the laminar flow cabinet to be exposed to UV light for 30 minutes as further sterilization.

The culture media used for the perpetual maintenance were MLA (14 December 2016 – 23 May 2017), Svetoslav medium (31 May 2017 – 31 July 2017), BBM (15 August 2017 – 20 January 2018), and BBMA medium (9 February 2018 – current). The culture media volume was 200 mL. An aseptic microbiological technique as written in Section 3.3. Starting a Culture was performed. Strain transfer into a new environmental microalgal culture was conducted every twenty days.

In Experiments 1 – 14, the culture was maintained in the OM11 Medium Orbital Shaker incubator at 60 µmol photons/m²s and 150 RPM. Later, the culture was kept in the MaxQ 6000 incubator at 25 °C and 150 RPM, and under continuous light at medium light intensity.

3.7 Harvesting Microalgal Culture and Dry Weight Measurement

The culture was harvested on day 14 of the cultivation. To collect biomass for further analysis, centrifugation was used to separate the cells from the culture medium. The culture was poured into a 50 mL Falcon tube and centrifuged at 2907 RCF for 10 minutes. The liquid was poured out of the tube, leaving the microalgae sediment at the bottom of the tube. The pellet was freeze dried and the tube weight was measured periodically until the weight was constant (approximately 5 days).

To measure the dry weight of the biomass, the culture was filtered using pre-weighed Munktell glass microfiber discs grade MGF (particle retention 0.7 µm) and dried to constant mass in an oven at 80° C (approximately 45 minutes).

3.8 Analysis

The fatty acid analysis was done by Callaghan Innovation, Wellington, New Zealand using gas-liquid chromatography (GLC) (Svetashev, Vysotskii, Ivanova, & Mikhailov, 1995). The repeatability was assessed by analysing samples from two flasks under identical conditions. The difference between the two analyses was insignificant (Table 8, 9, page 79).

Chapter IV

EFFECT OF DIFFERENT CULTURE MEDIA ON BIOMASS PRODUCTION OF MICROALGA *Trachydiscus* sp. LCR-Awa9/2

4.1. Introduction

It is acknowledged that under different growth conditions and culture media composition, the microalgae growth rate and biomass composition will change (Yeh & Chang, 2012). To determine which culture medium should be used as the basis culture medium for the culture media study, eight culture media that have been used for EPA producing microalgae were used to cultivate *Trachydiscus* sp. LCR-Awa9/2.

4.2. Methods

Trachydiscus sp. LCR-Awa9/2 culture was maintained in the OM11 Medium Orbital Shaker Incubator with a shaker speed of 150 RPM. It was grown in Svetoslav medium at room temperature, under continuous illumination using white fluorescent lamps at 60 µmol photons m^{-2} s^{-1}. The culture was cultivated for 19 days before it was inoculated into fresh culture media. The cell density for the inoculation process was 9.7×10^5 cells/mL.

Eight culture media was prepared for the experiment. The component mass is listed in Table 3. The New Medium by Svetoslav (Svetoslav medium) was prepared in two different total ionic concentrations, full strength Svetoslav medium (total ionic concentration 0.0592 M) and ¼ Svetoslav medium (total ionic concentration 0.0148 M).

18 flasks were prepared for the experiment with 9 culture media, each in duplicate. Each flask contained 100 mL of culture media. The initial cell density was 2×10^5 cells/mL. The cultivation process took place in the MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers incubator. The temperature was maintained at 25 °C. The culture was exposed to medium light intensity and shaken at 150 RPM. 3% CO_2 enriched air was supplied into the culture with a total mass flow rate for all flasks of 500 mL/min and a 0.2 µm filter was used to sterilize the gas.

The biomass concentration (g/L) was determined by filtration with pre-weighed Munktell glass microfiber discs grade MGF (particle retention 0.7µm) and drying to constant mass at 80 °C for approximately 45 minutes.

Table 3. Composition of various culture media used for growth of *Trachydiscus* sp. LCR-Awa9/2.

Component	**Mass (mg/L)**							
	MLAM	**BBM**	**BG11M**	**½SSM**	**OHM**	**MBM**	**SM**	**ZM**
$C_6H_{12}FeNO_7$			6		2.31			
NH_4VO_3						0.08		
H_3BO_3	2.4	11.42	2.86	3.09		0.16	2	6.18
$CaCl_2.2H_2O$	29.4	25	36	10.96	110.9	18.68	24.9	36.73
Cr_2O_3					7.6E-05			
$C_6H_8O_7$			6					
$CoCl_2.6H_2O$	0.01			1.36	1.1E-05	0.14	4E-03	2.73
$Co(NO_3)_2.6H_2O$		0.49	0.05					
$CuSO_4.5H_2O$	0.01	1.57	0.08	1.24	1.2E-05	0.03	0.4	2.49
$C_{10}H_{14}N_2NaFeO_9$		62.69		20.09		5.02		11.16
$Na_2C_{10}H_{14}N_2O_8.2H_2O$	4.56		1.27					
$(NH_4)_6Mo_7O_{24}.4H_2O$				1.84		0.09	0.04	1.96
$FeSO_4.7H_2O$						3.5	20	
$FeCl_3.6H_2O$	1.58							
$Fe_2(SO_4)_3.7H_2O$							20	
$MgSO_4.7H_2O$	49	75	75	988	246	30	266	25
$MnCl_2.4H_2O$	0.36	1.44	1.81		9.9E-04	4.95		
$MnSO_4H_2$				1.18			2	2.23
MoO_3		0.71						
KH_2PO_4		175		340				
$NiCl_2.6H_2O$						0.12		
$K_2HPO_4.3H_2O$	34.8	75	40			4	250	31
$KHNO_3$				2020	410	200		
SeO_2					5E-05			
H_2SeO_3	1.20E-03							
Na_2CO_3			20					21

Component	Mass (mg/L)							
	MLAM	**BBM**	**BG11M**	**½SSM**	**OHM**	**MBM**	**SM**	**ZM**
NaCl		25					50	
$NaHCO_3$	18					168	100	
$Na_2H_3PO_4$					30			
$NaMoO_4.2H_2O$	0.01		0.39		1.2E-04			
$NaNO_3$	170	250	1500				2000	467
$ZnSO_4.7H_2O$	0.02	8.82	0.22	1.43		0.36	2	2.87
$C_{12}H_{17}N_4OS.ClH.Cl$	0.1				0.02	0.05		
$C_{10}H_{16}N_2O_3S$	5E-04				0.03	0.33		
Vitamin B12	5E-04				0.02	0.05		

The summary of ionic concentration in the eight culture media that were tested in this experiment is given in Table 4.

Table 4. The ionic concentration of the culture media.*

Ion	Concentration (mol/L)							
	MLAM	**MBM**	**BBM**	**ZM**	**OHM**	**BG11M**	**SM**	**½ SSM**
Ammonium		1.08E-6		9.52E-6		1.13E-4	1.94E-7	8.93E-6
Boric	1.16E-4	7.52E-6	5.54E-4	1.00E-4		1.39E-4	3.24E-5	1.50E-4
Calcium	2.00E-4	1.27E-4	1.70E-4	5.00E-4	7.55E-4	2.45E-4	1.69E-4	7.46E-5
Carbonate	2.14E-4	2.00E-3		1.98E-4		1.89E-4	1.19E-3	
Chlorine	4.21E-4	3.05E-4	7.83E-4	2.73E-4	1.51E-3	5.08E-4	1.19E-3	1.61E-4
Chromium					1.00E-9			
Citrate					1.74E-5	7.64E-5		
Cobalt	4.20E-8	5.71E-7	1.68E-6	1.15E-5	4.63E-11	1.70E-7	1.68E-8	5.71E-6
Copper	4.01E-8	1.00E-7	6.29E-6	9.98E-6	4.81E-11	3.16E-7	1.60E-6	4.97E-6
EDTA	1.23E-5	1.30E-5	1.63E-4	2.90E-5		3.42E-6		5.22E-5
Iron	5.85E-6	2.56E-5	1.63E-4	2.90E-5	8.68E-6	2.26E-5	7.20E-5	5.22E-5
Magnesium	4.07E-4	2.49E-4	6.23E-4	2.08E-4	2.04E-3	6.23E-4	2.21E-3	8.21E-3
Manganese	1.82E-6	2.50E-5	7.28E-6	1.46E-5	5.00E-9	9.15E-6	1.31E-5	7.71E-6
Metavanadate		6.41E-7						
Molybdate	2.74E-8	7.28E-8	4.93E-6	1.59E-6	5.48E-10	1.79E-6	3.24E-8	1.49E-6
Nitrate	1.98E-3	1.96E-3	2.91E-3	5.43E-3	4.02E-3	1.74E-2	2.33E-2	1.98E-2
Phosphate	1.53E-4	1.75E-5	1.61E-3	1.36E-4	2.08E-4	1.75E-4	1.10E-3	2.50E-3
Potassium	3.05E-4	1.99E-3	1.94E-3	2.72E-4	4.02E-3	3.51E-4	2.19E-3	2.23E-2
Selenium	9.31E-9				4.51E-10			
Sodium	2.22E-3	2.01E-3	3.50E-3	5.86E-3	4.17E-4	1.78E-2	2.53E-2	5.22E-5
Sulphate	4.07E-4	2.63E-4	6.60E-4	2.42E-4	2.04E-3	6.24E-4	2.30E-3	8.22E-3
Zinc	7.65E-8	1.25E-6	3.07E-5	9.98E-6		7.72E-7	6.96E-6	4.97E-6
Total ionic concentration	**0.0064**	**0.009**	**0.013**	**0.013**	**0.015**	**0.038**	**0.059**	**0.062**

*Excluding vitamins

4.3. Results and Discussion

4.3.1 Repeatability

The legend in all graphs in this thesis includes a unique run identifier. All runs in this thesis were done in duplicate. The standard error of each run was mostly less than 15%. Standard errors more than 15% happened in some runs due to technical problems. As shown as an example in Figure 31, the difference between flask A and flask B of the microalga in Bold Basal Medium was minor. This small standard error was consistent in all runs. All graphs in this thesis are shown on the average of duplicates with error bars representing the standard deviation.

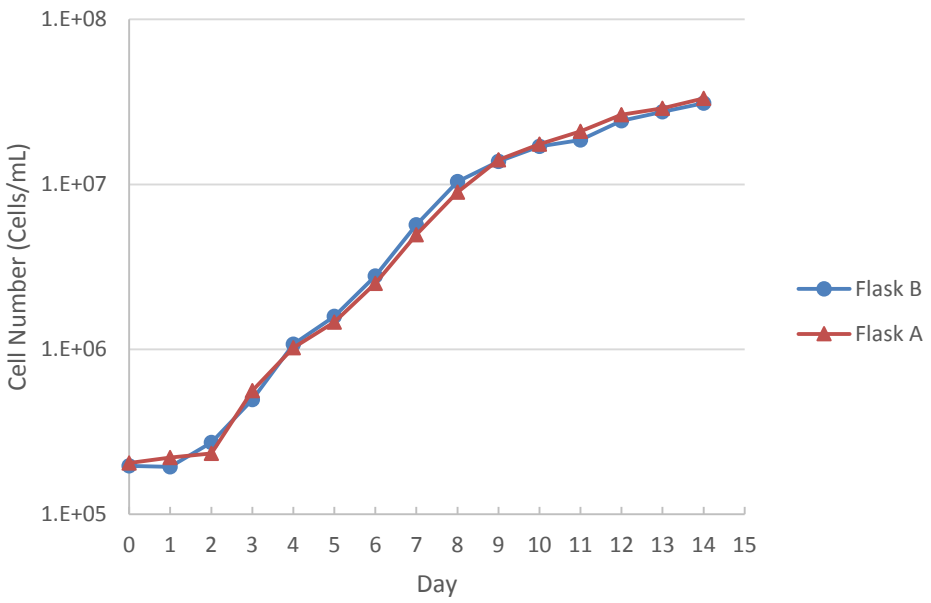

Figure 31. Typical example of duplicate result. The growth curve of *Trachydiscus* sp. LCR-Awa9/2 in Bold Basal medium in duplicate (Exp. 14.4).

4.3.2 The growth of *Trachydiscus* sp. LCR-Awa9/2 in different culture media

The experimental results of the growth of the microalga in different culture media are shown in Figure 32. The biomass production of the microalga in each culture medium is given in Figure 33. There were 2 days of lag phase in all culture media (except MLA, which had a lag phase of six days) because the inoculum was grown in different conditions than the experimental conditions, therefore the alga needed to adapt to new environmental conditions, such as changes in nutrient composition and culture conditions (temperature, light intensity, and gas sparging).

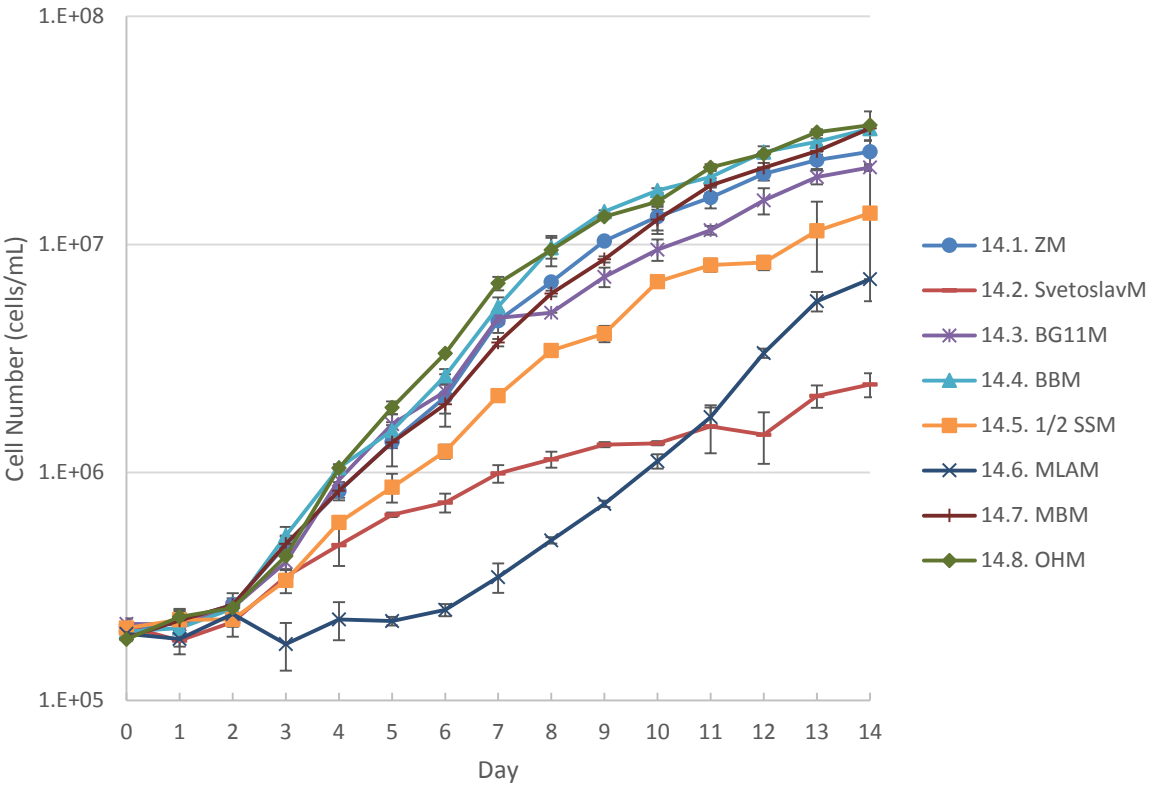

Figure 32. Growth curves of *Trachydiscus* sp. LCR-Awa9/2 in different culture media (Zehnder medium, Svetoslav medium, Blue Green 11 medium, Bold Basal medium, ½ Zachleder Setlik medium (SSM), MLA medium, Modified Bourrelly medium, and Optimum Haematococcus medium) under the same cultivation conditions (temperature, shaker speed, light intensity, 3% CO_2 enriched air flow mass). The legend includes a unique run identifier.

Selenium was suspected to be the cause of the extended lag phase in MLA culture. In a preliminary experiment (Appendix C, Experiment 7), when selenium was removed from MLA, the culture had a better growth rate than MLA with selenium in Experiments 5 and 6 (there was no significant growth until day 6). However, selenium was also used in OHM and in this experiment, and it performed the highest growth rate. The difference was that MLA used selenous acid (9.31×10^{-9} M) while OHM used selenium dioxide (4.51×10^{-10} M). There were two possible reasons for this result: first that the concentration of selenium in MLA was too high and inhibited the growth of the alga. Second, that different sources of selenium affected the culture growth. However, the second possibility does not agree with the theory that selenium dioxide diluted in water forms selenous acid (Wiberg, Wiberg, & Holleman, 2001).

$$H_2SeO_3 \rightleftharpoons SeO_2 + H_2O$$

Further investigation into the effect of selenium is included in Experiment 20 (Chapter VII).

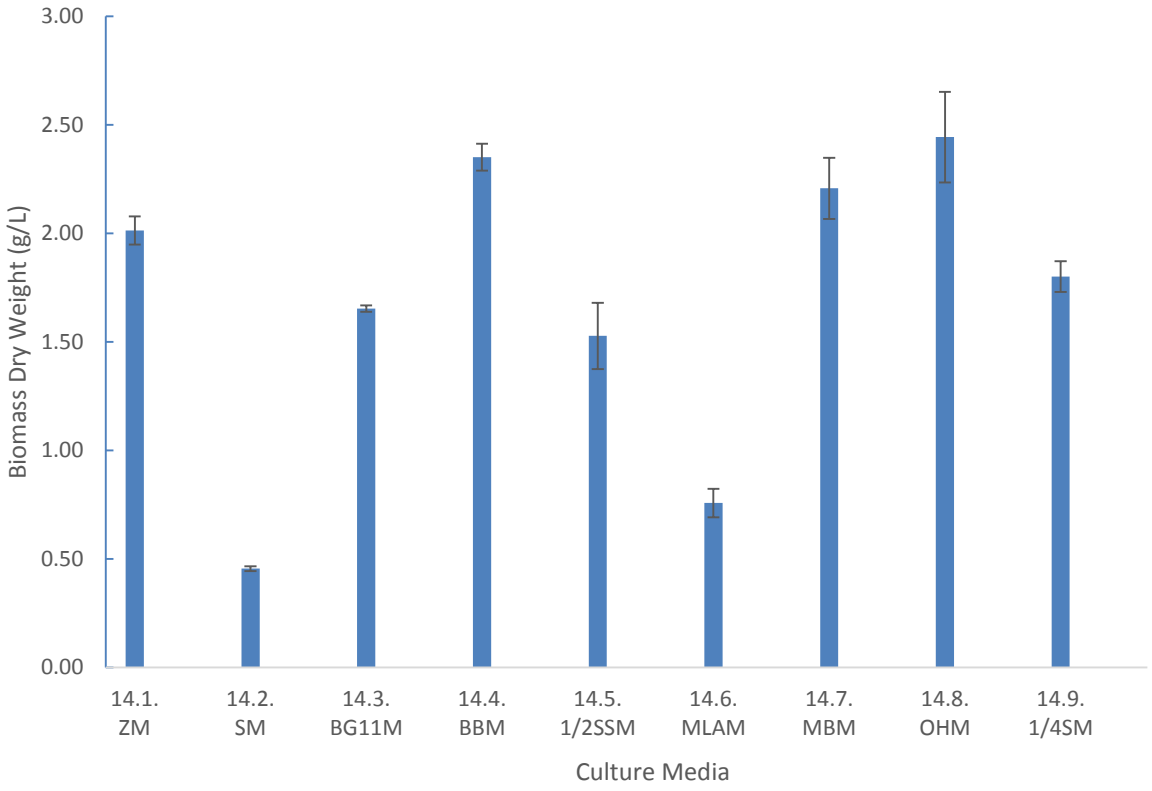

Figure 33. Biomass production performance of *Trachydiscus* sp. LCR-Awa9/2 in different culture media.

In terms of the growth curve which was expressed by cell density, OHM and BBM performed the best growth rates with slight differences. Their final cell number were 3.34×10^7 cells/mL and 3.2×10^7 cells/mL, respectively. The highest biomass dry weight, 2.4±0.2 g/L, was found in OHM grown culture, followed by BBM grown culture with 2.35±0.06 g/L (Figure 33). As a comparative example of another EPA producing microalgae, Freire et al. (2016) produced 1.8±0.2 g/L biomass of *Nannochloropsis limnetica* in OHM. The culture cell density was 2.5×10^8 cells/mL and the EPA content was close to 25%. It should be noted that *N. limnetica* size (2-6µm) is smaller than *T.* sp. LCR-Awa9/2 (6-9 µm). *N. limnetica* was also grown by Parupudi et al. (2016) in BBM for a CO_2 fixation study. The maximum biomass productivity was 0.072 g $L^{-1}d^{-1}$.

OHM and BBM were followed by ZM and MBM, where MBM started to outperform ZM in day 10. ZM produced a 2.5×10^7 cells/mL culture with 2.01±0.06 g/L dry biomass and MBM had a 3.2×10^7 cells/mL culture with 2.2±0.14 g/L dry biomass.

Zehnder medium is commonly used for *Trachydiscus minutus* cultivation. Řezanka et al. (2010), Řezanka et al. (2011), and Gigova et al. (2012) used ZM to grow the inoculum, which was then inoculated with the experimental culture media. Gigova et al. (2012) ran the experiment at 25 °C with a light intensity of 246 µmol $m^{-2}s^{-1}$ in "200 mL cultivating vessels". The initial density was 3×10^6 cells/mL, 15 times higher than the initial density in this experiment. After 4 days of cultivation, the culture had 3.1×10^7 cells/mL and produced 4.63

g/L biomass. In terms of cell density, this result is close to the highest gain in cell density in this experiment, but the biomass was almost twice as high.

Krienitz and Wirth (2006) used modified Bourrelly medium to study the influence of different culture conditions on the fatty acid content. The highest PUFA concentration was produced by a non-aerated suspension culture. It produced 55.56 mg PUFA/g DW, but the neither the dry weight nor the final cell density were given in the paper.

BG11 was used by Ma, Wang, Yu, Yin, and Zhou (2014) to grow *N. limnetica* for a biodiesel production study. BG11 gave a similar growth to ZM but became slower after day 7. Its final cell density was 2.2×10^7 cells/mL culture with 1.7±0.01 g/L dry biomass. MLA started to grow after day 6 and produced 0.76±0.07 g/L dry biomass from 7.1×10^6 cells/mL culture. ½SSM and SM gave the poorest growth with 1.4×10^7 cells/mL and 2.4×10^6 cells/mL; 1.5±0.15 g/L and 0.5±0.01 g/L dry biomass, respectively.

Thom (2015) cultivated *T.* sp. LCR-Awa9/2 in MLA and achieved 8.5×10^6 cells/mL cell concentration after 12 days. The experiment conditions were the same except that the culture was grown in an airlift photobioreactor with a light intensity of 140 µmol $m^{-2}s^{-1}$, and selenium was not used in MLA medium. There was no lag phase in Thom's (2015) experiment which could be a result of the absence of selenium.

Řezanka et al. (2010) and Cepák et al. (2014) used SSM for *T. minutus* studies (Cepàk, 2014 used half concentration). The initial cell density of both experiments was high, 4.2×10^7 cells/mL and 2.5×10^6 cells/mL, and produced 11.9 g/L and 12.28 g/L biomass, respectively.

Alexandrov et al. (2014) designed a new culture medium (this thesis refers to it as Svetoslav Medium or SM because the author did not name it) for *T. minutus*. The culture produced 7.8 g/L biomass.

The best four culture media had close ranges of total ionic concentration (0.013±0.0024 M), followed by BG11 with a total ionic concentration of 0.038 M, and SM and ½SSM with a range of total ionic concentration of 0.06±0.002 M. As shown in Figure 34, there appeared to a correlation between total ionic concentration and the growth rate. More evidence was given by Svetoslav medium in two different total ionic concentrations.

The total ionic concentration variations were tested because of the result from Experiments 9 and 13 (the data are in Appendix C). In Experiment 9, the microalga was cultivated in Svetoslav medium without CO_2 and the cell density at the end of the exponential phase (day 14) was up to 5×10^6 cells/mL with a growth rate of 0.19/day. A contrasting result was obtained from Experiment 13 where the microalgae was cultivated in the same condition as Experiment 9 but with 3% CO_2 in air supply. The cell density in day 14 was only 1.1×10^6 cells/mL with a growth rate of 0.13/day. The high total ionic concentration was suspected to be the cause of the growth rate reduction; therefore, the diluted Svetoslav medium was tested in this experiment to observe the effect of the total ionic concentration on the growth rate of the microalga.

When the SM was diluted into a quarter concentration (total ionic concentration 0.015 M), the growth rate was much higher than SM in its original total ionic concentration (Figure 35). ¼ SM growth came in the third position (after OHM and BBM) until day 10, then was overtaken by MBM and ZM afterwards. A similar result was obtained using BG11 (diluted to 0.006 M total ionic concentration) in Experiment 4 (Appendix C). It was hypothesised that the optimum total ionic concentration was about 0.013 M. However, this hypothesis was later disproved by Experiment 16 (Chapter V). The results of Experiment 16 showed that the inhibition did not come from the total ionic concentration, but probably form an individual ion concentration.

Figure 34

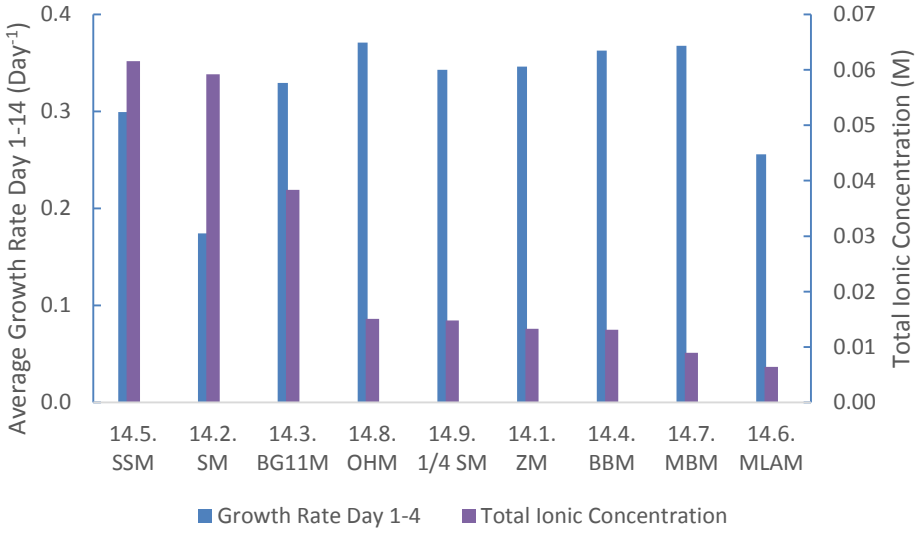

Figure 34. Average Growth rate (day 1-14) of *Trachydiscus* sp. LCR-Awa9/2 in different culture media plotted with each culture media total ionic concentration (TIC).

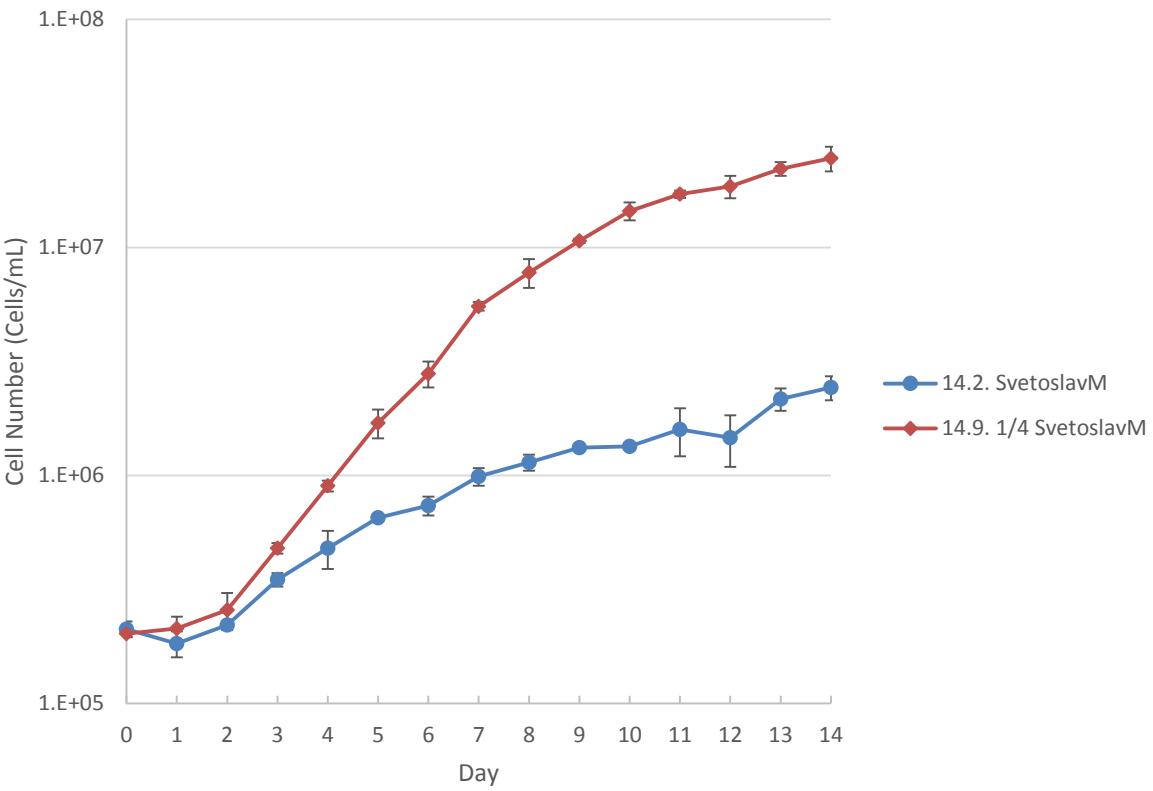

Figure 35. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in Svetoslav Medium with two different total ionic concentrations (Initial TIC and quarter TIC).

The cells' appearances in Figure 36 from SM and ½SSM (the high concentrate culture) were quite similar. The colour was pale and a lot of cell debris were seen. OHM and BBM cells were green and only few cell debris were seen. The cell debris might be because of the precipitation from the culture media. The more concentrated the culture media, the more the precipitation of minerals. SM and ½SSM had noticeable precipitation. The precipitation became worse after sterilization using the autoclave. The pale colour in SM and ½SSM might be caused by stress environment due to the toxic effect of an intolerable chemical concentration.

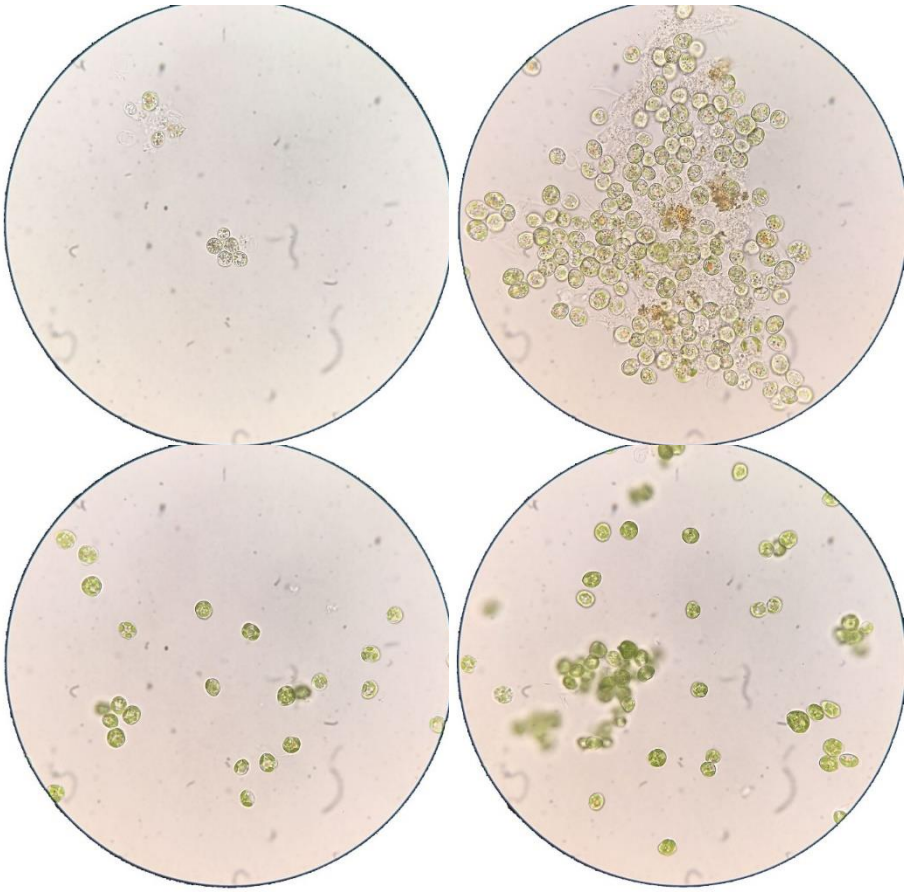

Figure 36. 8 days old *T.* sp. LCR-Awa9/2 cells in SM (top left), ½SSM (top right), BBM (bottom left), OHM (bottom right).

The macronutrient ions that were available in all culture media were (Table 4) nitrate, phosphate, potassium, sodium, sulphate, chlorine, and calcium. The popular micronutrient ions were EDTA, boron, cobalt, copper, iron, magnesium, manganese, molybdate, and zinc.

Both BBM and OHM each had 16 types of ions in the medium compositions. The differences were that BBM used boron and zinc while OHM did not, OHM was supplemented by chromium, selenium, and vitamins while BBM was not, and OHM used citrate as a chelating agent instead of EDTA. It appeared that chromium, selenium, and vitamins did not improve the growth rate significantly because BBM could achieve a similar growth rate, without these supplements.

4.4. Conclusions

OHM and BBM gave the best growth of *T.* sp. LCR-Awa9/2. The biomass productions were 2.44±0.21 g/L and 2.35±0.06 g/L, respectively. It seemed like there was a correlation between total ionic concentration and growth performance. BBM was used as the base culture medium for the culture media study due to its performance and simplicity in comparison to OHM.

Chapter V

EFFECT OF TOTAL IONIC CONCENTRATION AND OSMOTIC PRESSURE AND LIMITING NUTRIENT EVALUATION IN BIOMASS PRODUCTION OF MICROALGA *Trachydiscus* sp. LCR-Awa9/2

5.1. Introduction

It is understood that the best nutrient concentrations and cultivation conditions are necessary to optimize the cell number. In this study, the effect of ionic and osmotic stress will be investigated, along with limiting the nutrients that affect microalgae growth. The effect of different nitrogen sources is also reported in this chapter.

5.2. Methods

The cultivation process took place in the MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers incubator and ran for 14 days. Each condition was performed in duplicate. Each flask contained 100 mL of culture media. The initial cell density was 2×10^5 cells/mL. The temperature was maintained at 25 °C. The culture was shaken at 150 rpm. 3% CO_2 enriched air was supplied into the culture with a total mass flow rate for all flasks of 500 mL/min and a 0.2 µm filter was used to sterilize the gas.

5.2.1 The effect of total ionic concentration and osmotic stress to *Trachydiscus* .sp LCR-Awa9/2 growth

Experiment 16 cultures were exposed to medium light intensity. Bold Basal medium was prepared in six different concentration (quarter, half, one, two, four, and six times concentration) in order to observe the effect of total ionic concentration, while BBM supplemented by sodium chloride in three different concentrations to examine the influence of osmotic pressure in the microalga growth. 0.41, 1.18, and 1.94 g/L sodium chloride was added to BBM to provide the same total ionic concentration as 2, 4, and 6 BBM.
The osmotic pressure was calculated using equation:

$$\pi = \frac{RT}{\overline{V}} \ln(1 - X) \quad (6)$$

Where R is 82.05781cm^3atm $mol^{-1}K^{-1}$, V is partial volume of water (18.07 cm^3mol^{-1}), T is temperature (K), and X is ions mol fraction (Bajraktari, Hélix-Nielsen, & Madsen, 2017).

Table 5. Total ionic concentration and calculated osmotic pressure of culture media in Experiment 16.

Culture medium	**BBM**	**¼BBM**	**½BBM**	**2 BBM**	**4 BBM**	**6 BBM**	**2 BBM - NaCl**	**4 BBM - NaCl**	**6 BBM - NaCl**
Run identifier	**16.3**	**16.1**	**16.2**	**16.4**	**16.5**	**16.6**	**16.7**	**16.8**	**16.9**
TIC (mol/L)	0.013	0.003	0.007	0.026	0.053	0.079	0.026	0.053	0.079
π (bar)	0.321	0.080	0.161	0.642	1.285	1.926	0.642	1.285	1.926

The biomass concentration (g/L) was determined by filtration with pre-weighed Munktell glass microfiber discs grade MGF (particle retention 0.7µm) and drying to constant mass at 80 °C.

5.2.2 The evaluation of limiting nutrient in *T.* sp. LCR-Awa9/2 growth

Experiment 17 was performed to investigate the limiting micronutrient in the culture media. The cultures were exposed to medium light intensity. A quarter concentration of BBM was used as the basis culture medium. Nine ¼ Bold Basal media were prepared, each was supplemented on day three with one of: sodium nitrate (0.025 g/100 mL), potassium chloride (0.0145 g/100 mL), sodium ferric EDTA (0.006 g/100 mL), magnesium sulphate (0.0075 g/100 mL), boric acid (0.00114 g/100 mL), calcium chloride (0.0025 g/100 mL), monopotassium phosphate (0.0175 g/100 mL), and disodium sulphate (0.001 g/100 mL). The concentration of each chemical was the same as the normal BBM recipe. Below are the list of culture media conditions that were tested in the experiment:

17.1. Quarter concentration of BBM (¼BBM)
17.2. ¼ BBM that was supplemented by $NaNO_3$
17.3. ¼ BBM that was supplemented by KCl
17.4. ¼ BBM that was supplemented by Fe-Na-EDTA
17.5. ¼ BBM that was supplemented by $MgSO_4$
17.6. ¼ BBM that was supplemented by H_3BO_3
17.7. ¼ BBM that was supplemented by $CaCl_2$
17.8. ¼ BBM that was supplemented by KH_2PO_4
17.9. ¼ BBM that was supplemented by Na_2SO_4

The biomass concentration (g/L) was determined by filtration with pre-weighed Munktell glass microfiber discs grade MGF (particle retention 0.7µm) and drying to constant mass at 80 °C.

5.2.3 The effect of different sources of nitrogen to *T.* sp. LCR-Awa9/2 growth

The preferred nitrogen source by the microalga was then tested by comparing growth in culture media that used nitrate, urea, and ammonium. These culture media were prepared with the same nitrogen concentration, 0.006 M. 2BBM with urea as the nitrogen source, and that was supplemented by nickel chloride was also prepared to investigate the role of nickel in the cultivation process. The basis culture media was BBM2N. The cultures were exposed to medium light intensity. Experiment 20 conditions were:

20.1. Control medium: BBM2N (N source: $NaNO_3$)
20.2. 2BBM with Urea

20.3. 2BBM with Urea+$NiCl_2$ (Ni concentration 5×10^{-7} M)
20.4. 2BBM with Ammonium chloride

Another experiment was performed as a confirmation. The cultures were grown under high light intensity exposure. The culture medium used in this experiment was the final prototype medium (Chapter VII). Experiment 23 conditions were:
23.1. Control medium: BBMA* (N source: $NaNO_3$)
23.8. BBMA with Urea+$NiCl_2$
23.9. BBMA with Ammonium chloride
*) BBMA is the final prototype of culture medium that have been developed in this project for *T.* sp. LCR-Awa9/2.

The dry mass was determined by centrifugation at 2907 RCF for 15 minutes and freeze drying to constant mass for approximately 5 days. Fatty acid analysis was done by Callaghan Institute to show the effect of different nitrogen sources to the microalga fatty acid composition.

5.3. Results and Discussion

5.3.1 The effect of total ionic concentration and osmotic stress to *Trachydiscus* sp. LCR-Awa9/2 growth

Unlike Experiment 14 in the previous chapter, the growth curve of all cultures did not show any lag phase. This was because the inoculum was grown in the same conditions and culture media as the experiment, so the cells were able to quickly adapt to a new culture.

The effect of salinity on *T.* sp. LCR-Awa9/2 growth rate was tested by growing the microalgae in BBM in different concentrations of NaCl (Figure 37). The concentration of NaCl was varied in order to make the total ionic concentrations the same as 2, 4, and 6 BBM total ionic concentration. 0.409, 1.176, and 1.944 g/L NaCl was added to BBM to make 2, 4, and 6 BBM-NaCl supplemented, respectively. No significant differences were found between the control and the tested NaCl supplemented culture medium. The dry mass of cells was also closely comparable to the control. This result disproves the previous hypothesis that TIC affects the growth of the culture media. Although 6BBM-NaCl had high total ionic concentration, 0.079 M, the culture grew better than 6BBM. According to this result, a concentration of NaCl up to 1.94 g/L was tolerable for the microalga. Determination of the limiting concentration of NaCl was beyond the scope of this study.

Trachydiscus minutus was strongly inhibited when grown in a culture medium with 0.6 g/L NaCl. The tolerable range of NaCl for *T. minutus* was between 0 – 0.6 g/L. However, the highest biomass and lipid were achieved by a culture medium without NaCl (Cepák et al., 2014).

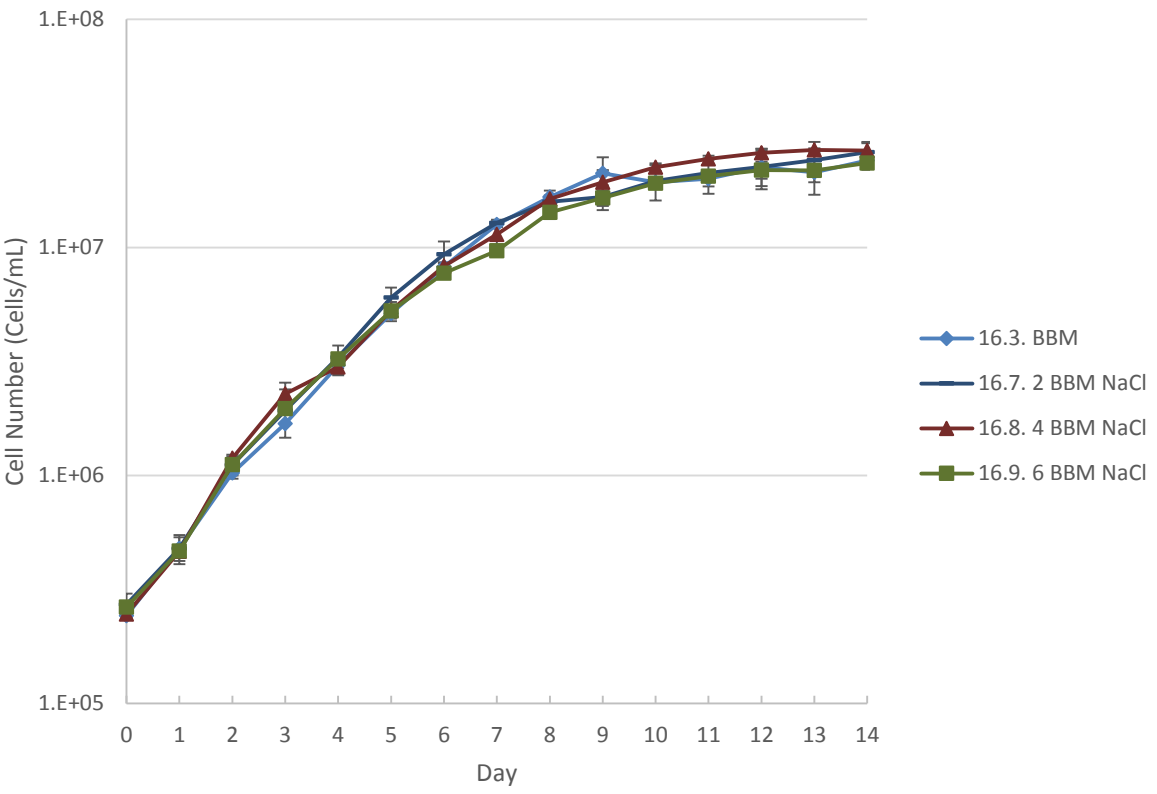

Figure 37. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in Bold Basal Medium in different osmotic pressure.

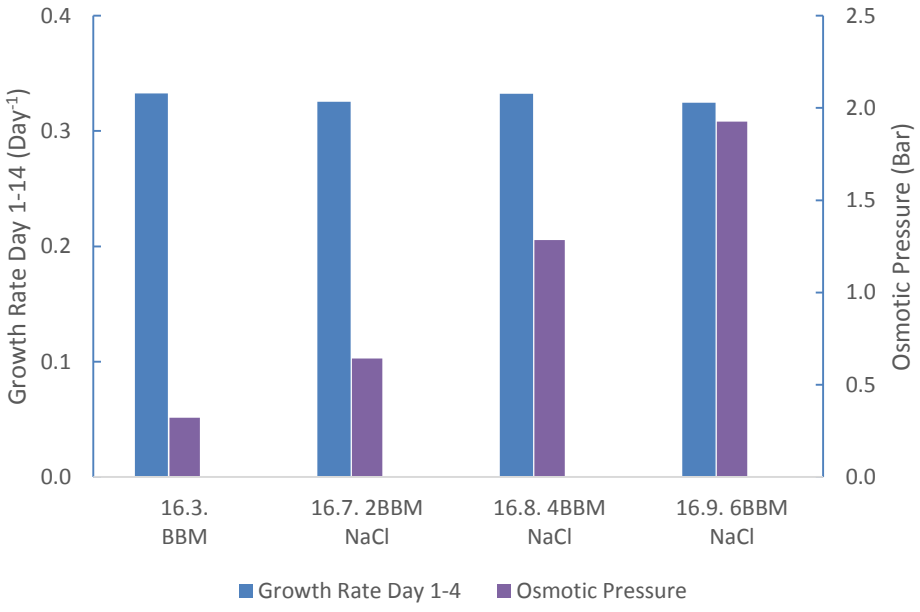

Figure 38. Growth rate of *Trachydiscus* sp. LCR-Awa9/2 in Bold Basal Medium with different osmotic pressure.

The growth of *Trachydiscus* sp. LCR-Awa9/2 in different total ionic concentrations of BBM increased equally with each addition except the highest concentration culture medium (Figures 39, 40, and 41). The poorest growth rate was obtained by ¼BBM culture. The growth rate improved when the total ionic concentration of the culture media was increased and all showed

similar growth rates until day 5, except 6 BBM. 2BBM and 4BBM showed similar growth until the end of the cultivation. The growth dropped significantly when the six times culture medium was used. This result disproved the hypothesis in Chapter IV that total ionic concentration affects the growth of the microalga. 2BBM and 4BBM had slight differences in growth and dry biomass production and the total ionic concentration of 4BBM concentration was almost as high as SM and ½SSM. It was clear that it is the concentration of individual chemicals that plays a role in the microalga cultivation. When ¼BBM, ½BBM, and BBM became depleted of one or more nutrients, they entered the starvation phase in sequence. ¼BBM entered the stationary phase on day 5, ½BBM on day 8, and BBM on day 10. There were one or more chemicals that passed the maximum concentration tolerated by the microalga, so growth was inhibited from early in the growth phase.

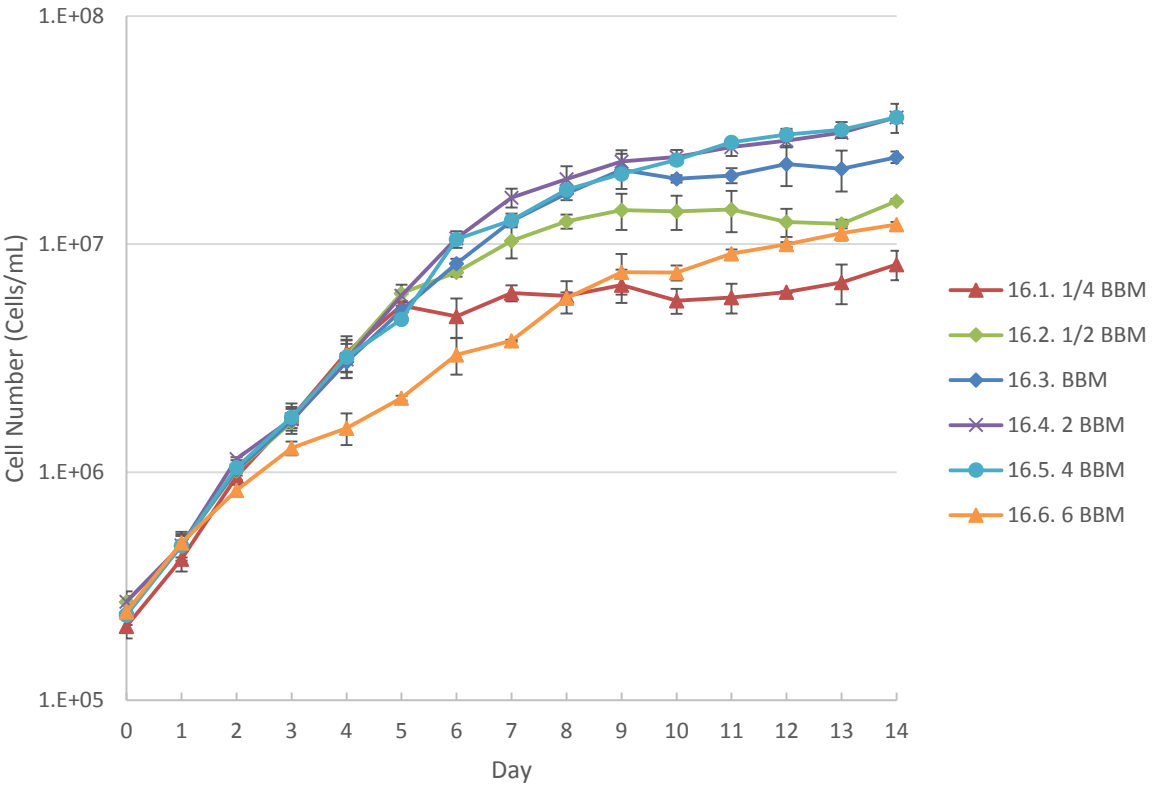

Figure 39. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in Bold Basal Medium in various total tonic concentrations (TIC).

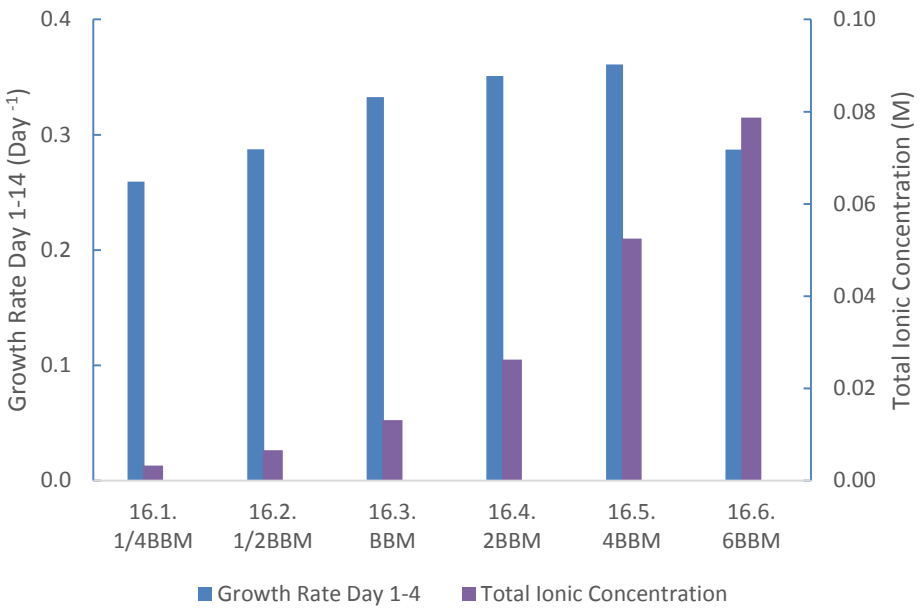

Figure 40. Average growth rate of *Trachydiscus* sp. LCR-Awa9/2 in Bold Basal Medium with various total ionic concentration.

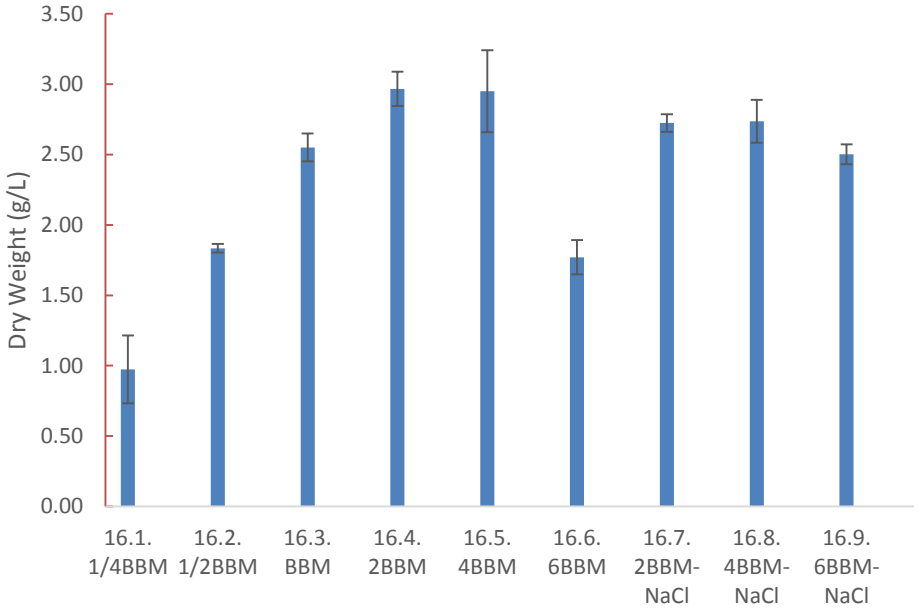

Figure 41. Biomass production performance of *Trachydiscus* sp. LCR-Awa9/2 in Bold Basal Medium with various total ionic concentration and osmotic pressure.

As shown in Figure 41, the biomass production corresponded to the growth curve. ¼BBM, ½BBM, BBM, 2BBM, 4BBM, and 6BBM produced 0.97±0.24, 1.83±0.03, 2.55±0.1, 2.97±0.12, 2.95±0.29, and 1.77±0.12 g/L, respectively. Like the growth curve, 2, 4, and 6 BBM NaCl biomass production have small differences.

Chen et al. (2011) grew Trachydiscus minutus in Zehnder medium in three different concentrations, Z (0.0133 M), 3Z (0.04 M), and 6Z (0.08 M). Z medium showed the poorest growth rate with 3 g/L dry biomass. 3Z growth was closely comparable to 6Z with 4 g/L dry

weigh. By comparing 6 BBM and 6 ZM in table 4, it is noted that 6 BBM has much higher phosphate (11.9×), potassium (7.2×), EDTA and iron (5.6×), borate (5.5×), zinc (3.1×), molybdate (3.1×), magnesium (3×), chlorine (2.9×), and sulphate (2.7×). Therefore, any or all of these might be the inhibition. This was not pursued further in the experiment. Phosphate and potassium in 6 BBM have no toxic effect (Kim et al., 2013; Talling, 2010). Borate concentration in 6 BBM (0.003 M) might have exceeded the alga tolerance. Fábregas et al. (2000) reported that *Haematococus pluvialis* had more growth when removing borate from the culture medium (the control medium used 0.0001 M boric acid). *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, and *Cladophora* growth were inhibited with zinc concentration was higher than 1.7×10^{-5} M (Cao et al., 2015; Zhou et al., 2012), while in 6 BBM zinc concentration was 1.84×10^{-4} M. The concentration of sulphate in 6 BBM, 0.004 M, was higher than Mera et al. (2016) sulphate optimal range for *Chlamydomonas moewusii* which was 0.0001 – 0.0003 M. Therefore, borate, zinc, and sulphate in 6 BBM might be at toxic level. Chlorine in 6 BBM at a concentration of 0.033 M was not the inhibitor since the alga survive in 6 BBM – NaCl (Experiment 16.9).

5.3.2 The evaluation of limiting nutrient in *T.* sp. LCR-Awa9/2 growth

The next experiment was performed in order to investigate the limiting of nutrients that inhibits the growth in ¼, ½, and 1 BBM. The base culture medium was ¼BBM. On day 3, each flask was supplemented with $NaNO_3$, KCl, Fe-Na-EDTA, $MgSO_4$, H_3BO_3, $CaCl_2$, KH_2PO_4, and Na_2SO_4.

From the growth curve (Figure 42), it was clear that N was the main limiting nutrient. Culture media that were supplemented with Fe-Na-EDTA, $MgSO_4$, KH_2PO_4, and Na_2SO_4 showed similar growth to the control. KCl and $CaCl_2$ were slightly lower than the control, while H_3BO_3 addition had a toxic effect on the culture. The growth curve was consistent with the dry weight (Figure 43). The control biomass dry weight was 1.01±0.08 g/L.

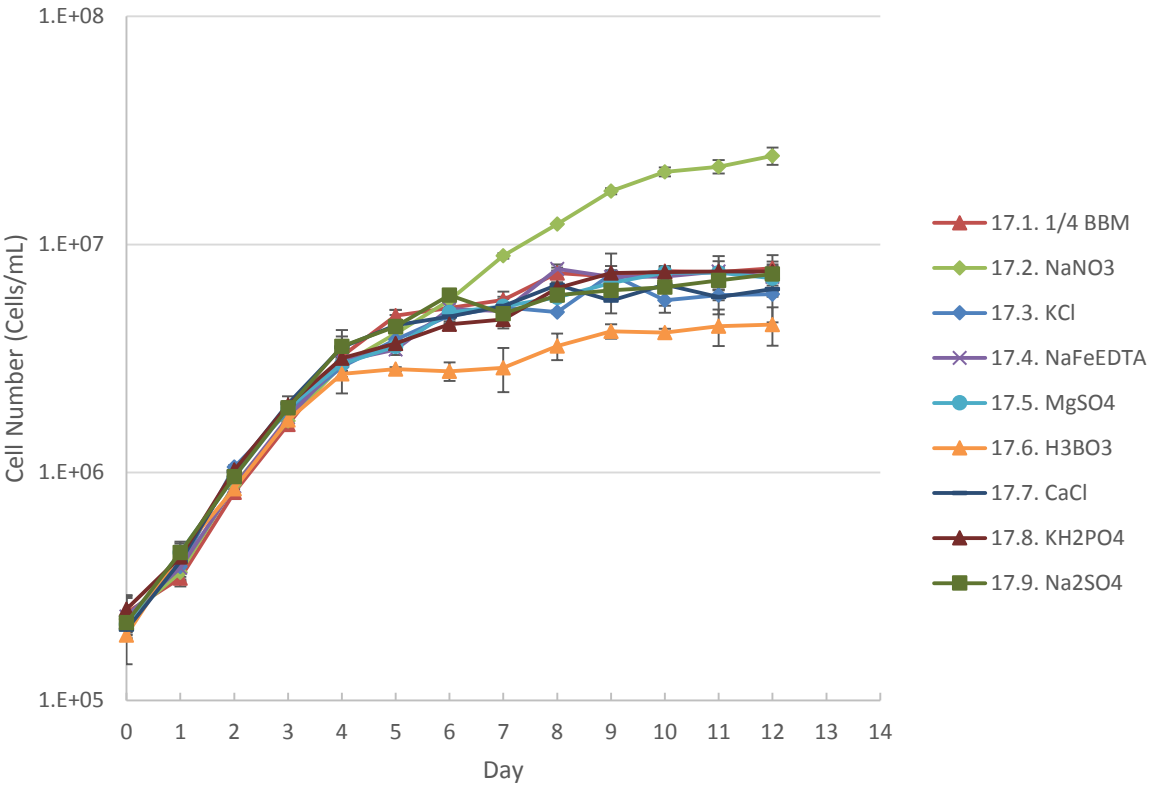

Figure 42. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in supplemented ¼ Bold Basal Medium.

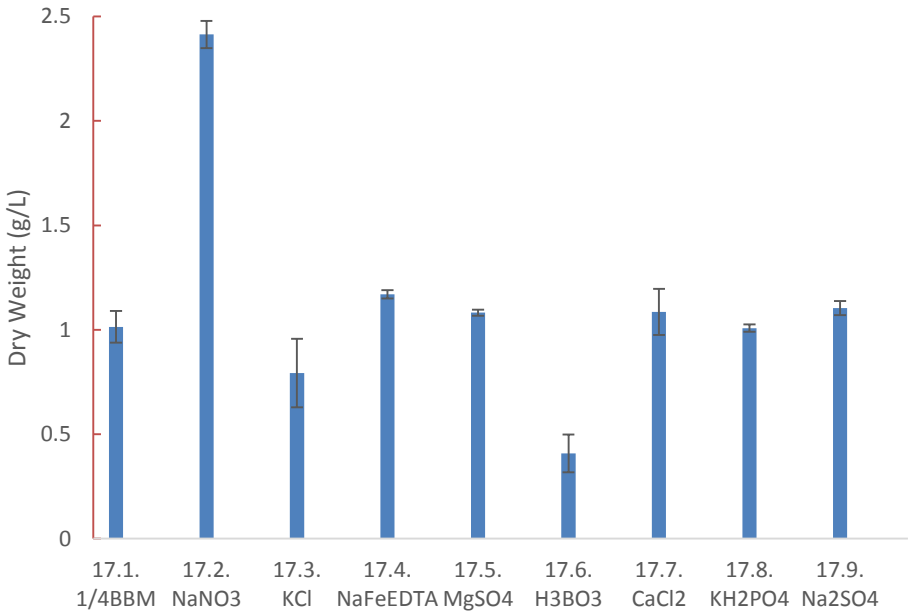

Figure 43. Biomass production performance of *Trachydiscus* sp. LCR-Awa9/2 in supplemented ¼ Bold Basal Medium

The addition of sodium sulphate to the culture did not produce considerable change from the control. After adding the chemical, the sulphur concentration in the media altered from 0.00087 M to 0.00153 M. The final sulphur concentration was lower than the maximum tolerance of

sulphur in *C. moewusii* which was 0.003 M (Mera et al., 2016). The culture produced 1.1±0.03 g/L dry weight.

In this experiment, boron appeared to have a toxic effect as the culture growth became lower than the control after the concentration of boron was increased from 0.00014 M to 0.0007 M on day 4. Fábregas et al. (2000) also found that boron is toxic for *Haematococcus pluvialis*. 0.0006 M concentration of boron was used for *H. pluvialis* culture, but a higher cell density was obtained when it was removed from the culture. *T.* sp. LCR-Awa9/2 only produced 0.41±0.09 g/L dry biomass in boron supplemented culture.

The experiment started with a Fe(III)Na-EDTA concentration of 0.00004 M, which was then increased to 0.0002 M on day 4. The culture growth was similar to the control medium with only minor differences. The dry biomass that was obtained from the culture was 1.8±0.02 g/L. Iron was not the limiting nutrient which starved the growing culture.

Similar to Fe-Na-EDTA addition, the Mg supplemented culture growth curve was similar to the control. The initial concentration was 0.00016 M and it was then altered to 0.0008 M. 1.08±0.01 g/L dry biomass was obtained from the culture.

T. sp. LCR-Awa9/2 culture produced less biomass than the control when the control was supplemented with KCl (0.0019 M) and $CaCl_2$ (0.0002 M). The biomass production was 0.79±0.16 g/L and 1.09±0.11 g/L, respectively. The presence of abundant chloride seemed to provoke oxidative stress in the culture (Liu, Wang, & Zhou, 2008).

A concentration range of phosphate from 0.0004 M to 0.065 M seemed adequate for *T.* sp. LCR-Awa9/2 as the culture showed similar growth as the control. The modelling of phosphate requirement will be discussed in the next chapter.

5.3.3 The effect of different sources of nitrogen to *T.* sp. LCR-Awa9/2 growth

Nitrogen is known as the most essential nutrient required by microalgae. Microalgae can assimilate N from various sources. This experiment investigated the effect of different nitrogen sources on *T.* sp. LCR-Awa9/2 growth. There were three nitrogen sources that were tested: nitrate from sodium nitrate, ammonium from ammonium chloride, and urea. All cultures had the same amount of nitrogen which was 0.006 M. One of the urea cultures that was supplemented with nickel as nickel is present in the enzyme urease. The enzyme is necessary for nitrogen uptake in culture media that use urea as their nitrogen source (Sunda et al., 2005).

As shown in Figure 44, there was a day of lag phase in the urea and ammonia culture, indicating that the cell needed to adapt to new chemicals. During the exponential phase, the ammonia culture growth was higher than the other cultures. It entered an early stationary phase on day 10. Using urea as a nitrogen source was promising as it gave the best result. The urea culture that was supplemented by nickel grew better than the one without nickel. This result was consistent with a report by Price and Morel (1991) which showed that nickel stimulates the enzyme urease activity which facilitates the nitrogen assimilation.

During the exponential phase, the nitrate growth rate in medium growth was higher than in urea media. In day 10, urea+Ni medium outnumbered the nitrate medium, and so did the urea medium in day 14. The growth curve corresponded with the biomass productions. Urea+Ni culture produced the highest dry biomass, 2.6±0.4 g/L (Figure 45). Urea culture gained 2.4±0.08 g/L and nitrate culture produced slightly less than urea medium, 2.4±0.2 g/L. Ammonia culture had the least dry biomass production, which was 1.7±0.2 g/L. This results corresponded to Cepák et al. (2014) and Alkhamis and Qin (2015) as described in Chapter II.

Alexandrov et al. (2014) established a new culture medium for *T. minutus* by comparing the new culture medium with two different nitrogen sources which were sodium nitrate and urea. The nitrate culture worked better than the urea culture. It produced 7.8 g/L while urea culture only produced 6.76 g/L dry biomass.

Similar to this study, Jin-Liu, Sommerfeld, and Hu (2013) reported that nitrate and urea fed cultures of *Isochrysis galbana* shared almost identical growth and DHA production in 10 days of cultivation. The least production was shown by ammonium culture which was attenuated after day 4. The growth reduction in ammonium culture was suspected to be caused by pH reduction along with the cellular uptake of ammonia. In this experiment, the pH of ammonia culture dropped from 7 to 3.13. The alga culture pH always dropped when entering the stress conditions such as nitrogen or phosphate starvation, but they had never dropped to be less than 5.87.

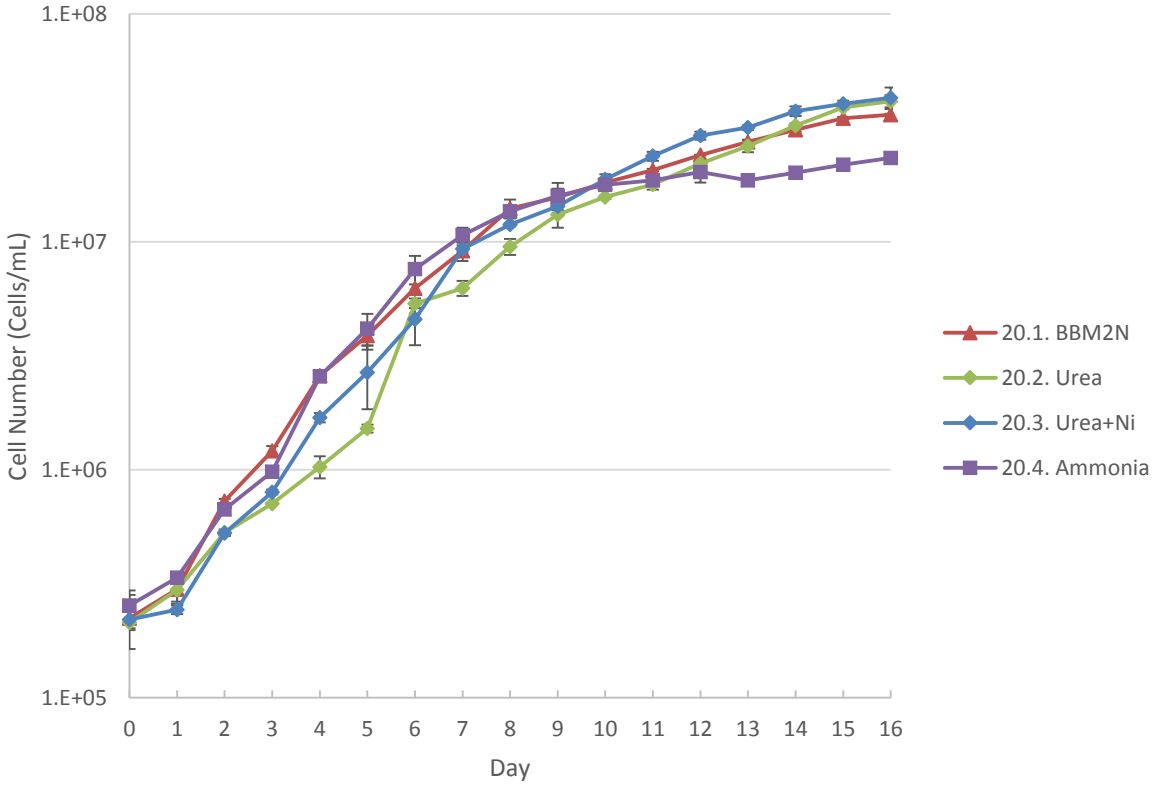

Figure 44. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in BBM2N media with different sources of nitrogen.

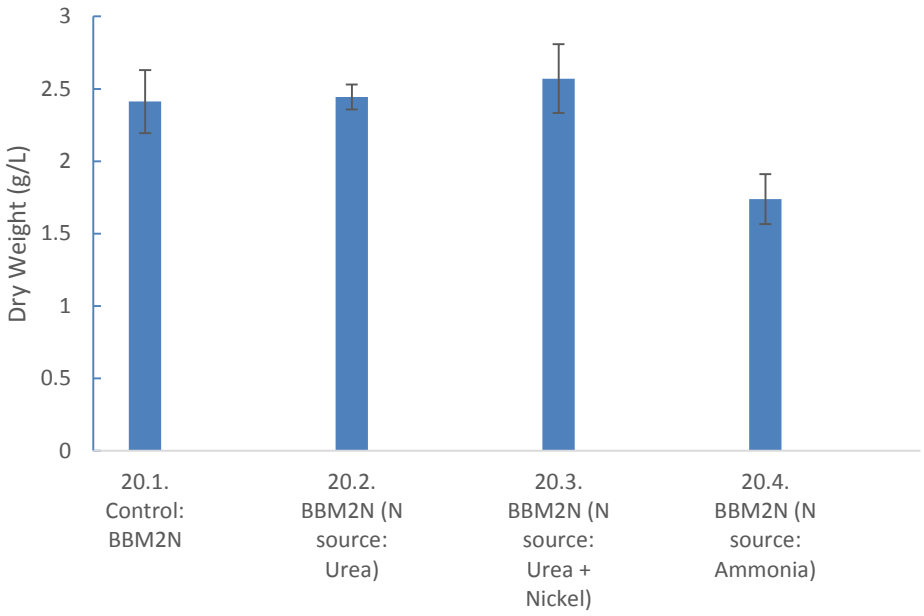

Figure 45. Dry biomass production from *T.* sp. LCR-Awa9/2 grown in BBM2N media with different sources of nitrogen.

Another experiment was performed to confirm the result of Experiment 20 (Figure 46). The biomass productions both in cell number and dry biomass were higher than Experiment 20 because of two improvements: high light intensity (the light inhibition is discussed in Chapter VII) and BBMA medium. The growth of three different conditions was similar to Experiment 20, but in this experiment the nitrate source culture medium performed better than the urea+Ni culture. This might be because of removing cobalt and boron from the culture. In Experiment 20, the basis culture medium still used cobalt and boron so they might inhibit the cell growth. Like Experiment 20, the ammonia source culture medium gave the poorest growth.

Unlike Experiment 20, there was no lag phase in urea+Ni culture because the inoculum was also from urea+Ni culture. Like Experiment 20, NH_4^+ culture did not have lag phase. The final pH of NH_4^+ culture was 6.2, much higher than the final pH in Experiment 20. This could be caused by a gas distribution problem (the detail is given in Chapter VII, page 103) resulting in less CO_2 being sparged into the flask.

The final cell number of the control medium was 8.1×10^7 cells/mL and the dry biomass was 4.71±0.12 g/L (Figure 47). In term of biomass production, sodium nitrate was the most favourable nitrogen source for the microalga.

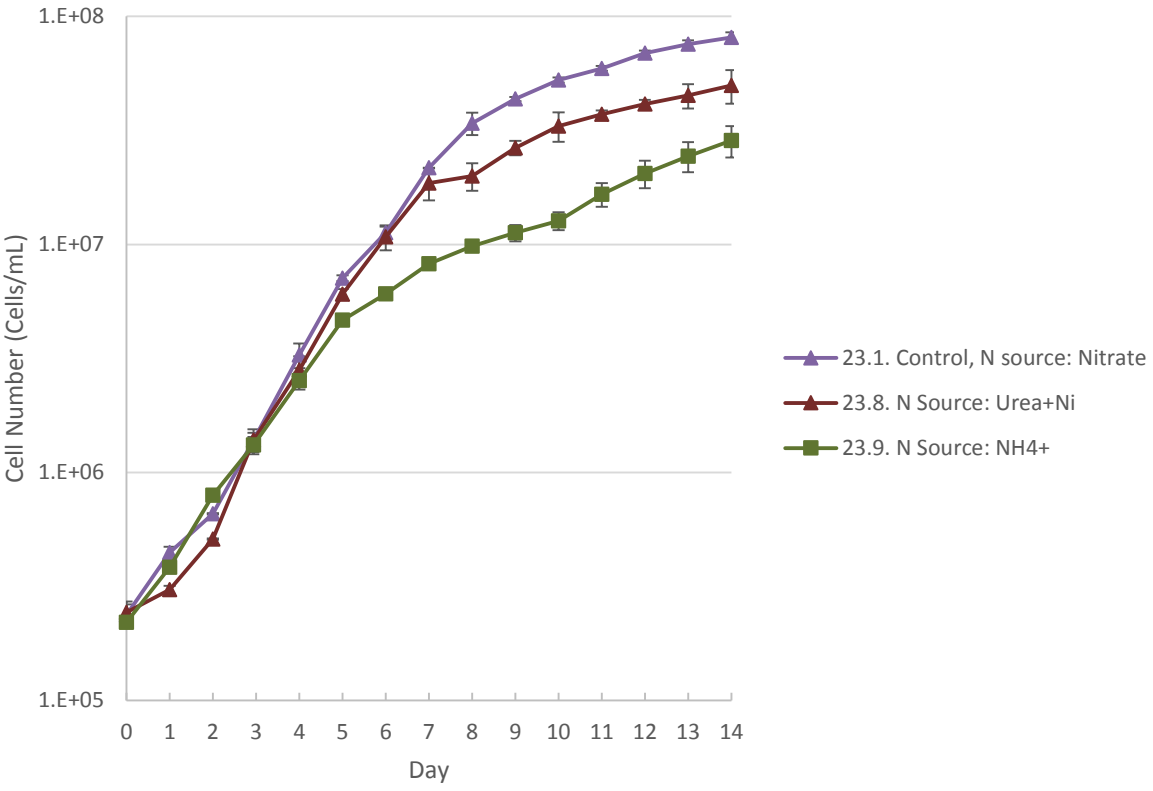

Figure 46. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in BBMA medium with different sources of nitrogen.

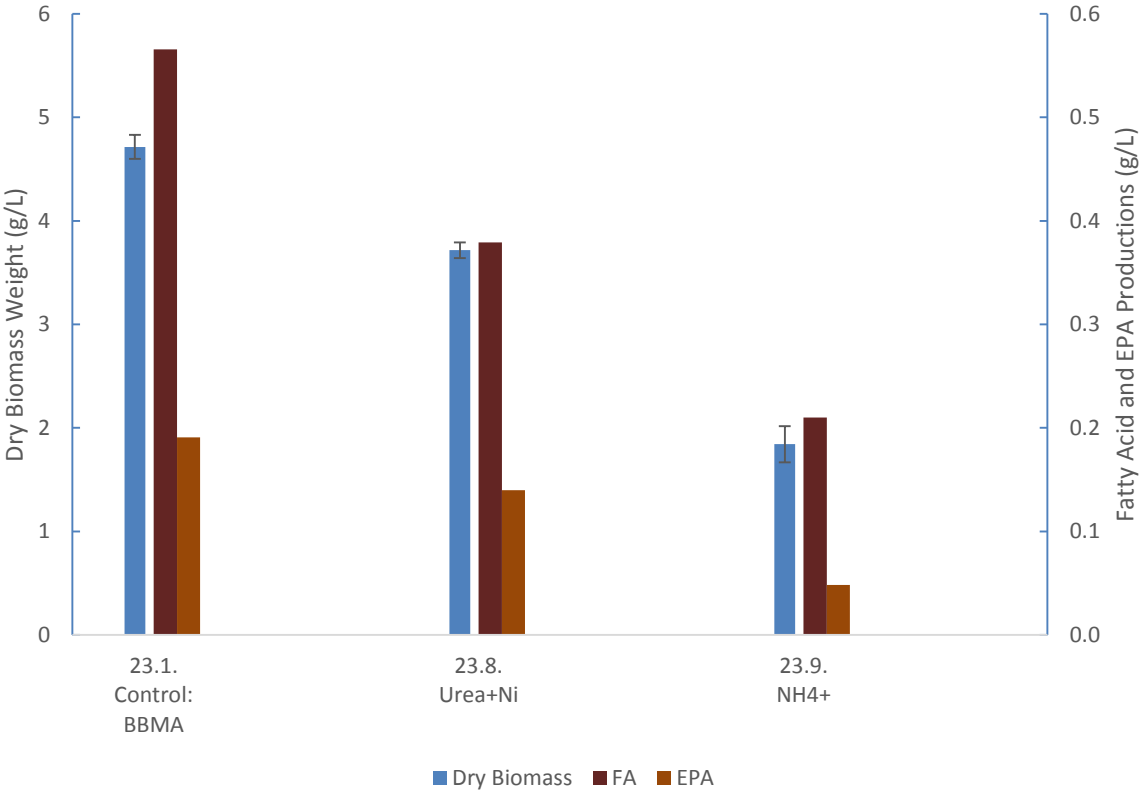

Figure 47. Dry biomass production from *T.* sp. LCR-Awa9/2 grown in BBMA medium with different sources of nitrogen.

The fatty acid profile from the different sources of nitrogen cultures are given in Table 6 and 7. The nitrate culture (the control medium) produced the highest concentration fatty acid as a property in dry matter, which was 12%, followed by NH_4^+ and urea+Ni cultures with 11.4% and 10.2% fatty acid in dry mass (FA in DM), respectively. The highest EPA value was obtained by the nitrate culture which was 4.05 g/100 g FA or 33.7% of total FA. The urea culture stimulated more production of myristoleic acid, 16:02, α-linolenic acid, and arachidic acid than nitrate and NH_4^+ culture. The NH_4^+ culture produced more myristic acid, palmitic acid, stearic acid, elaidic acid, linoleic acid, and DGLA.

Cepák et al. (2014) reported that *T. minutus* produced 21.24% lipid in dry biomass (DM) and 22.5±3.3% EPA of total FA in a culture medium with KNO_3 as the nitrogen sources. The urea culture produced slightly higher lipids and EPA than the nitrate culture, 21.35% lipid in DM and 22.5±1.2% EPA of total FA. Similar to Experiment 20 and 23, the NH_4^+ culture of *T. minutus* gave the lowest lipid and EPA production. The $NH_4^+NO_3$ culture produced 18.39% lipid in DM and 19.69±0.63% EPA of total FA, and $(NH_4)_2CO_3$ culture produced 16.38% lipid in DM and 20.67±1.79% EPA of total FA.

Table 6. The effect different sources of nitrogen on fatty acid profile in *T.* sp. LCR-Awa9/2 (Exp. 23).

Fatty Acid g/100g Total FA	**23.1. Control: BBMA**	**23.8. Urea+Ni**	**23.9. NH_4^+**
Myristic acid (14:0)	16.50	17.34	20.65
Myristoleic acid (14:1n-5)	0.63	1.21	0.46
Palmitic acid (16:0)	7.62	7.21	8.32
Palmitoleic acid (16:1n-7)	10.10	7.43	7.67
16:02	0.48	0.68	0.11
Stearic acid (18:0)	1.23	0.43	1.35
Elaidic acid (18:1n-9)	3.64	1.39	9.71
Vaccenic acid (18:1n-7)	0.18	0.15	0.13
Linoleic acid (18:2n-6)	6.69	6.49	10.61
α-Linolenic acid (18:3n-3)	2.17	4.00	1.73
Arachidic acid (20:0)	0.54	0.16	0.52
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.55	0.49	0.80
Arachidonic acid (20:4n-6)	3.44	6.61	4.22
Eicosapentaenoic acid (**20:5n-3**)	33.75	36.86	23.01
Behenic acid (22:0)	7.22	2.05	6.10
Total fatty acids g/100 g dry biomass	**12.00**	**10.20**	**11.40**

Table 7. The fatty acid composition in 100 g dry mass (Exp.23).

FA g/100 g DM	23.1. Control: BBMA	23.8. Urea+Ni	23.9. NH4+
Myristic acid (14:0)	1.98	1.77	2.35
Myristoleic acid (14:1n-5)	0.08	0.12	0.05
Palmitic acid (16:0)	0.91	0.74	0.95
Palmitoleic acid (16:1n-7)	1.21	0.76	0.87
16:02	0.06	0.07	0.01
Stearic acid (18:0)	0.15	0.04	0.15
Elaidic acid (18:1n-9)	0.44	0.14	1.11
Vaccenic acid (18:1n-7)	0.02	0.02	0.02
Linoleic acid (18:2n-6)	0.80	0.66	1.21
α-Linolenic acid (18:3n-3)	0.26	0.41	0.20
Arachidic acid (20:0)	0.07	0.02	0.06
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.07	0.05	0.09
Arachidonic acid (20:4n-6)	0.41	0.67	0.48
Eicosapentaenoic acid (**20:5n-3**)	4.05	3.76	2.62
Behenic acid (22:0)	0.87	0.21	0.70
FA g/L	**0.57**	**0.38**	**0.21**
EPA g/L	**0.19**	**0.14**	**0.05**
g EPA/g dry biomass	**0.04**	**0.04**	**0.03**
Dry Mass (g/L)	**4.71**	**3.72**	**1.84**

5.4. Conclusions

The total ionic concentration did not affect the growth of *Trachydiscus* sp. LCR-Awa9/2, but the concentration of individual chemicals did. Each element has a tolerance range for the microalga nutrient uptake, therefore finding it is necessary for culture medium design. The alga seemed to tolerate sodium chloride up to 2 g/L.

The limiting nutrient in BBM was nitrogen. 0.5 – 1 g/L sodium nitrate seemed to be enough for 14 days cultivation. Further experiments and modelling for the amount of nitrogen required for the microalgae was performed and will be discussed later.

Among the three most common nitrogen sources, nitrate was the most favourable for the microalga. The highest biomass production in this section was achieved by BBMA in Experiment 23, with 4.71 g/L dry biomass, 12% FA of DM, and 33.75% EPA of total FA.

Chapter VI

EVALUATION OF NITROGEN AND PHOSPHATE STARVATION IN THE MICROALGA *Trachydiscus* sp. LCR-Awa9/2

6.1. Introduction

It is well known that microalgae will develop secondary metabolites in stressed environments. This chapter reports experimental results about the effect of nitrogen and phosphate starvation in the microalga *Trachydiscus* sp. LCR-Awa9/2. A dynamic model on the growth of the microalga is also shown in this chapter. The model was developed by Weaver (2017). The model was run based on light and nutrient (nitrogen and phosphorous) requirements. Through the modelling and simulations, the amount of nitrogen and phosphorous required by the microalga was determined (in g nutrient/g biomass).

6.2. Methods

Three inoculum cultures was prepared, 2BBM4N for control, ½N culture for nitrogen depleted culture, and $^{1}/_{10}$P culture for phosphate depleted culture.

Each condition was performed in duplicate. Each flask contained 100 mL culture media. The initial cell density was 2×10^5 cells/mL. The cultivation process took place in the MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers incubator. The temperature was maintained at 25 °C. The culture was exposed to high light intensity and shaken at 150 RPM. 3% CO_2 enriched air was supplied into the culture with a total mass flow rate for all flasks of 500 mL/min and a 0.2 µm filter was used to sterilize the gas.

A low nitrate culture medium and a low phosphate culture medium were prepared. The low nitrate culture medium was ½ times lower than N concentration in original BBM, while the low phosphate culture was $^{1}/_{40}$. The experiment conditions were:
22.3. 2BBM4N harvested in day 7
22.8. Control: 2BBM4N
22.5. 2BBM½N harvested after cell density reached 1×10^7 cells/mL
22.4. 2BBM½N
22.7. 2BBM$^{1}/_{40}$P harvested after cell density reached 1×10^7 cells/mL
22.6. 2BBM$^{1}/_{40}$P

Another experiment was done to investigate more about the microalga growth under starved conditions. BBMA (refer to Chapter VII) was used as the control medium. The alga was grown in nitrogen, phosphate, and complete starvation conditions.

In one of the ½N culture medium, 0.0105 M of sodium nitrate was added on day 10. For complete starvation, after 12 days of cultivation, the culture was centrifuged at 7000 RPM for 10 minutes and the cells were washed twice using sterile MiliQ water. Then the cells were transferred into the a containing sterile MilliQ water with the same volume as the previous culture. The culture then sat in the incubator for three more days before harvesting. Experiment 23 conditions were:

23.1. BBMA
23.3. BBMA½N
23.4. BBMA½N harvested on day 10
23.5. BBMA½N + 3.5N on day 10
23.6. BBMA $^{1}/_{40}$P
23.7. BBMA-Complete starvation

The biomass was harvested by centrifugation at 2907 RCF for 15 minutes. The liquid was disposed then the samples were dried using freeze dryer. The dry samples were sent to Callaghan Innovation for fatty acid analysis.

6.3. Result and Discussion

Based on Experiment 16 in Chapter V, it was clear that nitrogen was the limiting nutrient that inhibited culture growth. The nitrogen concentration needed to be increased to at least two times higher in order to prevent nitrogen starvation in the 14 days of cultivation. When the culture started to starve of nitrogen, the culture became difficult to homogenise. *T.* sp. LCR-Awa9/2 produced a sticky gelatinous material under nitrogen starvation conditions which might cause them to remain attached or form clumps more readily than at other times (Phil Novis, personal communication, October 23, 2017). This phenomenon did not appear in phosphate starvation culture and micronutrient depleted cultures (Chapter VII). All cultures in the nutrient starvation experiments had similar growth from day 0 until day 5. The first sign of starvation occurred on day 5. This included the cell growth reduction and change in colour.

One of the obvious signs of starvation was the change in colour as shown in Figure 48 and 49. The colour of a healthy culture was fresh green and the green colour became darker with the increasing cell density. When the nitrogen starvation began, the culture colour changed to pale green. Nitrogen starvation causes a substantial reduction in the photosynthesis activity, which is related to significant changes in pigmentation, generally chlorophyll content reduction and a rise in carotenoid levels (Shehawy & Diethelm, 2001). This colour change phenomenon is known as chlorosis or bleaching (Gigova & Ivanova, 2015).

Figure 48. *T.* sp. LCR-Awa9/2 culture from Experiment 16. Top: Culture with nitrogen starvation. Bottom: Healthy culture.

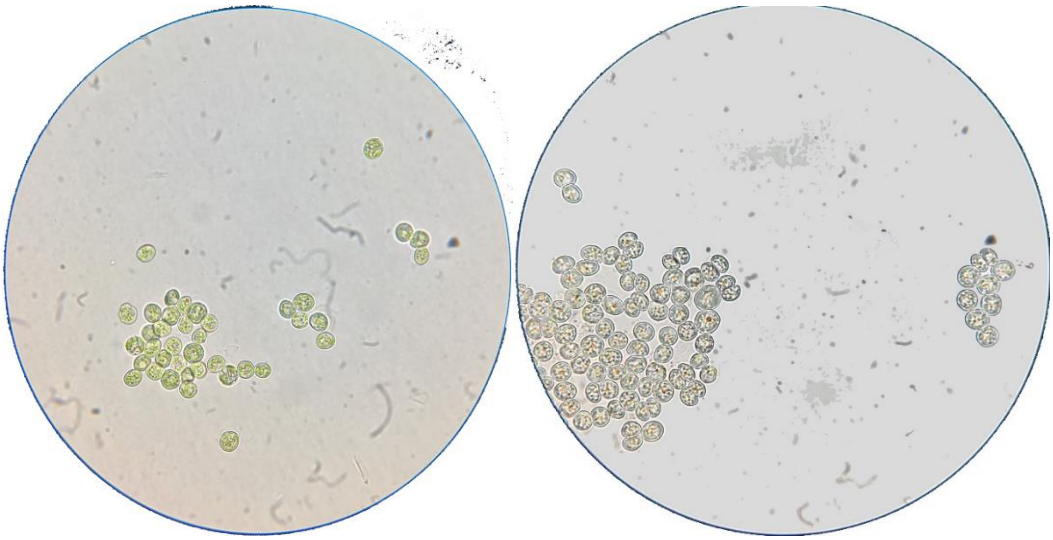

Figure 49. *T.* sp. LCR-Awa9/2 cells in N replete culture (left) and N depleted culture (right)

As shown in Figure 50, under N-depleted and P-depleted conditions, the microalgae biomass productivity was lower than the control. The N-depleted culture only achieved 2.15±0.01 g/L and P-depleted 1.72±0.01 g/L dry biomass. The control displayed the highest biomass production at 3.82±0.13 g/L.

In three days of cultivation, *Trachydiscus minutus* in a full strength of culture medium produced 0.85±0.04 g/L dry biomass. The production was reduced in the N-depleted culture to 0.69±0.03g/L (Gigova & Ivanova, 2015).

Maximum daily biomass productivity of N and P starvation cultures was obtained in the first 6 days of cultivation (Figure 50 and 51). During this period, the N-depleted, P-depleted, and the control showed similar cell density accumulations. The N-depleted and P-depleted cultures exhibited a lower growth from day 6.

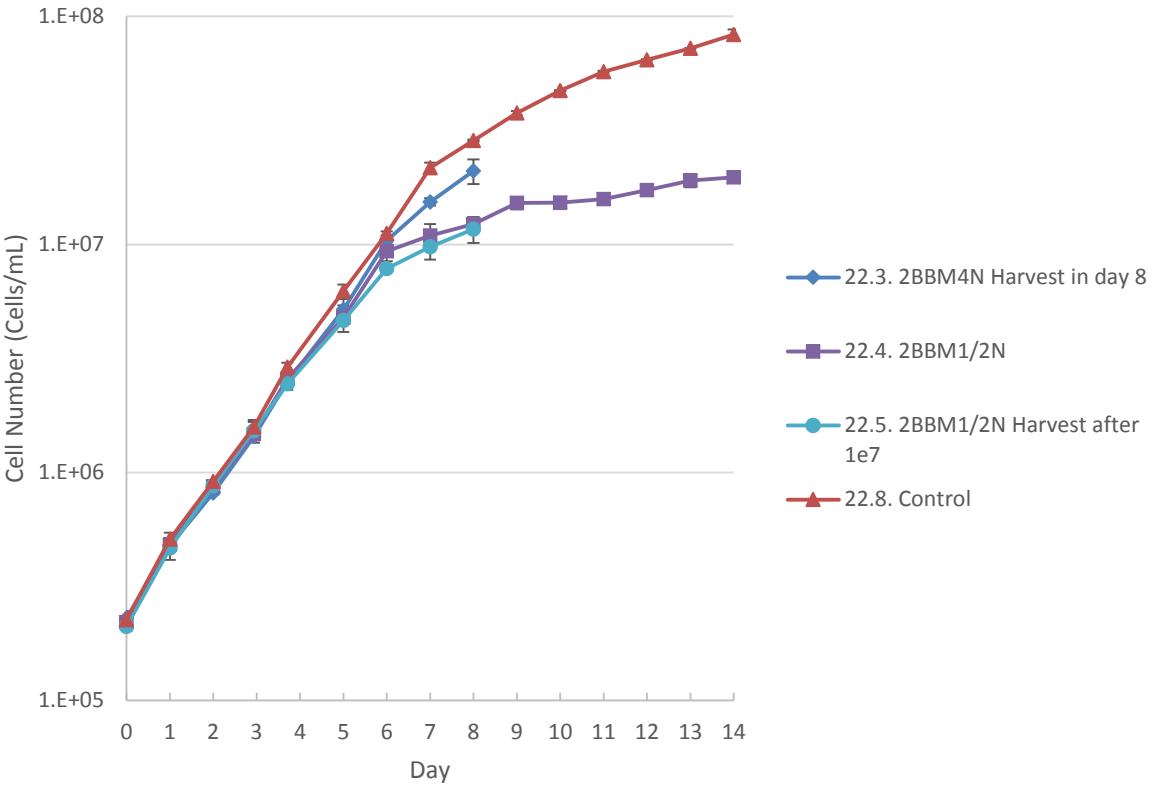

Figure 50. The effect of N starvation to *T.* sp. LCR-Awa9/2 growth.

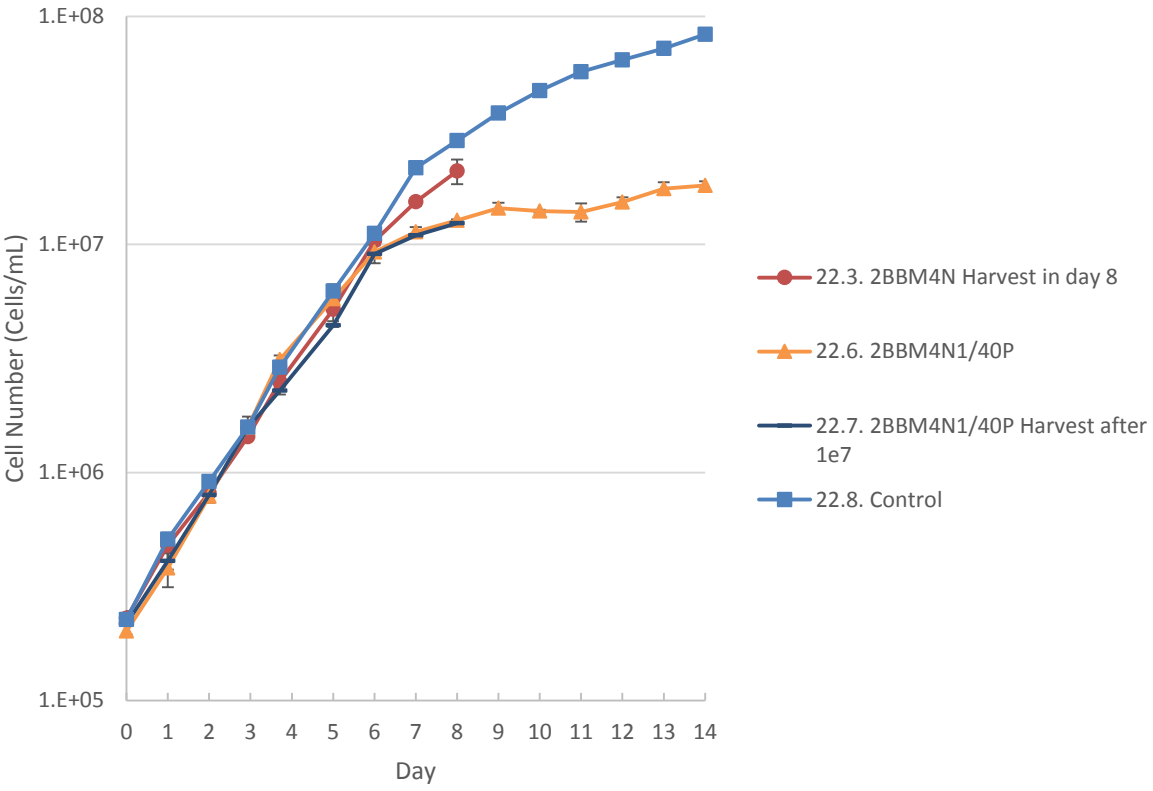

Figure 51. The effect of P starvation to *T.* sp. LCR-Awa9/2 growth.

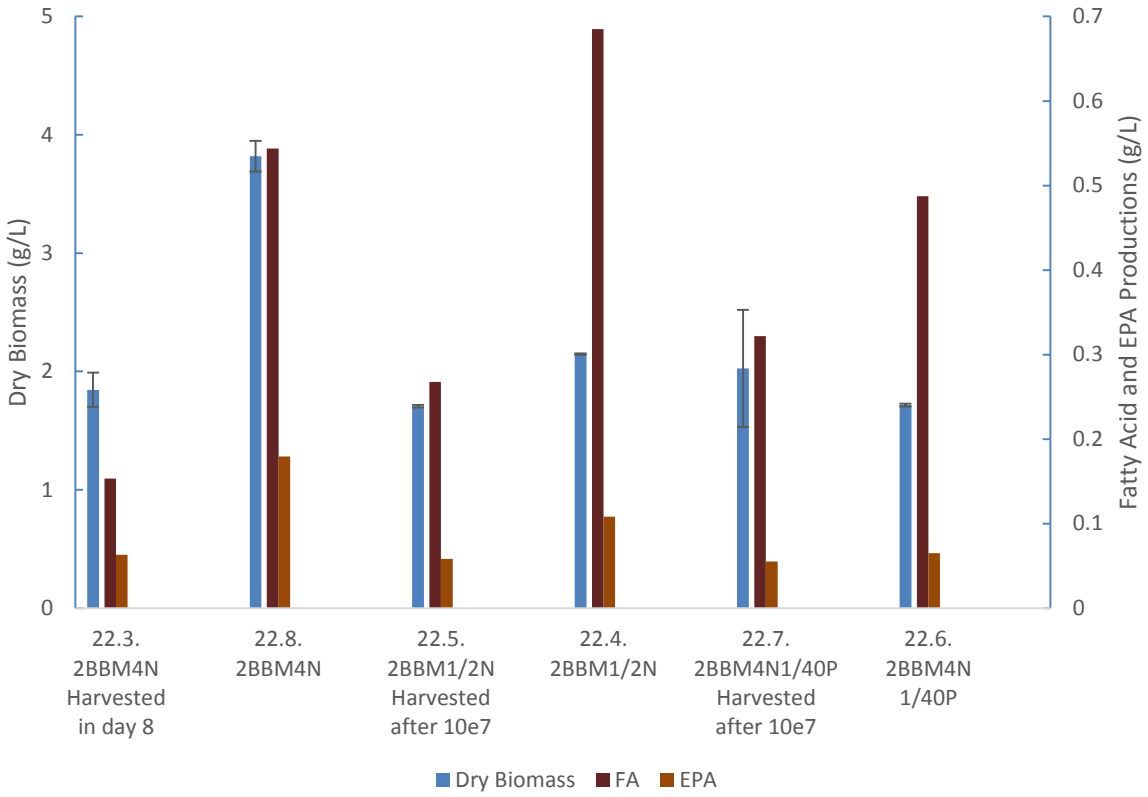

Figure 52. The effect of N and P starvation to biomass, fatty acids, and EPA production of *T.* sp. LCR-Awa9/2.

Under N starvation conditions, the culture produced the highest percentage of lipid in DM at 32%, followed by P-depleted culture at 28% and the control medium at 14% (Table 8).

To investigate the potential of EPA production, the fatty acid profiles under N-depleted and P-depleted cultures were analysed. Different harvesting time were applied in this experiment to observe the optimum harvesting time. The results are shown in Table 8 and 9.

Table 8. The effect of N and P starvation on fatty acid profile in *T.* sp. LCR-Awa9/2 (Exp.22).

Fatty Acid, g/100g DM	22.3. 2BBM4N Harvested in day 8	22.8. 2BBM4N	22.8. 2BBM4N	22.5. 2BBM½N Harvested after 10e7	22.4. 2BBM½N	22.7. 2BBM4N $^{1}/_{40}$P Harvested after 10e7	22.6. 2BBM4N $^{1}/_{40}$P
Myristic acid (14:0)	1.52	2.36	2.33	3.16	6.79	4.25	7.79
Myristoleic acid (14:1n-5)	0.03	0.03	0.03	0.03	0.06	0.03	0.11
Palmitic acid (16:0)	0.62	1.09	1.07	1.21	2.28	1.11	1.52
Palmitoleic acid (16:1n-7)	0.54	1.43	1.30	1.83	4.01	1.96	3.80
16:2	0.02	0.06	0.05	0.02	0.02	0.04	0.06
Stearic acid (18:0)	0.05	0.21	0.19	0.26	0.55	0.22	0.32
Elaidic acid (18:1n-9)	0.28	0.52	0.48	2.26	6.22	1.92	4.35
Vaccenic acid (18:1n-7)	0.01	0.02	0.02	0.02	0.03	0.03	0.04
Linoleic acid (18:2n-6)	0.33	0.99	0.95	0.89	2.49	0.72	1.97
α-Linolenic acid (18:3n-3)	0.19	0.28	0.29	0.18	0.23	0.34	0.64
Arachidic acid (20:0)	0.02	0.07	0.06	0.05	0.11	0.04	0.08
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.07	0.10	0.10	0.17	0.23	0.46	0.69
Arachidonic acid (20:4n-6)	0.31	0.47	0.49	0.32	0.54	0.40	0.81
Eicosapentaenoic acid (**20:5n-3**)	3.43	4.58	4.51	3.40	5.04	2.72	3.78
Behenic acid (22:0)	0.34	0.97	0.86	1.40	2.59	0.88	1.44
FA g/100g DM	8.3	13.9	13.5	15.7	31.9	15.9	28.4
FA g/L	0.153	0.544	0.503	0.268	0.685	0.322	0.487
EPA g/L	0.063	0.179	0.168	0.058	0.108	0.055	0.065
Dry Mass g/L	1.845	3.912	3.728	1.706	2.148	2.025	1.716

Table 9. The fatty acid profiles fraction of total fatty acid (Exp.22).

Fatty Acid g/100g Total FA	22.3. 2BBM4N Harvest in day 8	22.8. 2BBM4N	22.8. 2BBM4N	22.5. 2BBM½N Harvested after 10e7	22.4. 2BBM½N	22.7. 2BBM4N $^{1}/_{40}$P Harvested after10e7	22.6. 2BBM4N $^{1}/_{40}$P
Myristic acid (14:0)	18.29	17.01	17.30	20.15	21.28	26.76	27.44
Myristoleic acid (14:1n-5)	0.31	0.22	0.23	0.16	0.20	0.22	0.40
Palmitic acid (16:0)	7.52	7.85	7.91	7.74	7.13	6.97	5.36
Palmitoleic acid (16:1n-7)	6.51	10.26	9.61	11.67	12.57	12.32	13.39
16:2	0.27	0.40	0.34	0.11	0.06	0.24	0.20
Stearic acid (18:0)	0.64	1.51	1.40	1.63	1.73	1.38	1.13
Elaidic acid (18:1n-9)	3.42	3.71	3.59	14.40	19.51	12.10	15.32
Vaccenic acid (18:1n-7)	0.16	0.16	0.15	0.13	0.09	0.19	0.15
Linoleic acid (18:2n-6)	3.96	7.14	7.03	5.70	7.80	4.52	6.94
α-Linolenic acid (18:3n-3)	2.30	1.98	2.16	1.12	0.72	2.16	2.24
Arachidic acid (20:0)	0.28	0.49	0.47	0.33	0.35	0.26	0.28
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.81	0.70	0.73	1.09	0.72	2.90	2.43
Arachidonic acid (20:4n-6)	3.68	3.40	3.65	2.01	1.69	2.50	2.84
Eicosapentaenoic acid (**20:5n-3**)	41.30	32.97	33.42	21.66	15.79	17.08	13.31
Behenic acid (22:0)	4.15	6.96	6.37	8.94	8.12	5.52	5.07
Total	93.61	94.77	94.36	96.82	97.76	95.12	96.50

The alga that was grown in the control medium produced 4.5 g EPA/100 g dry biomass in 14 days of cultivation. The EPA content was 33% of the total fatty acids and it reduced from the 41.3% that was achieved in 8 days of cultivation. During the last 6 days of cultivation, the lipid percentage increased from 8% to 14%, along with an increase in palmitic acid, palmitoleic acid, 16:2, stearic acid, elaidic acid, linoleic acid, arachidic acid, and behenic acid. The accumulation of palmitoleic acid, linoleic acid, and behenic acid was the most significant. The percentage of vaccenic acid was constant, while the other fatty acids were decreased slightly, with the exception of EPA. Based on the growth curve, the end of the exponential phase occurred on day 9. When the cells entered the stationary phase, which can be caused by limited light or nutrients, they started to accumulate other fatty acid but not EPA.

Řezanka et al. (2010) reported that *T. minutus* produced 15.4% EPA of total fatty acid in nitrogen and sulphur replete ½SS culture medium. A higher percentage of EPA, 22.57% of FA, was achieved by Cepák et al. (2014) when the alga was grown in ½SS medium with urea as its nitrogen source. The fraction of EPA in fatty acid increased to 37% when the concentration of Zehnder medium was doubled (Řezanka et al., 2015). The maximum production of EPA was found to reach 51% of the total fatty acid when the Z medium was supplemented by 5.1 mg/L iron nanoparticles nanofer 25 (Pádrová et al., 2015).

Under nitrogen starvation conditions, the culture produced more fatty acid than the control. However, the amount of EPA was less than the control medium, only 15.8% of total fatty acid. The culture accumulated more elaidic acid (19.51%), myristic acid (21.28%), and palmitoleic acid (12.57%). Other fatty acids were also slightly higher than the control medium, such as stearic acid, linoleic acid, dihomo-gamma-linolenic acid (DGLA), and behenic acid.

The fatty acid production in 14 days of cultivation under nitrogen starvation conditions increased twice as much as in 8 days of cultivation under the same conditions. The transformation in the fatty acid components were a major increase in elaidic acid, linoleic acid, and myristic acid, and a minor increase in palmitoleic acid, stearic acid, myristoleic acid, and arachidic acid. The other fatty acids were reduced, including EPA, which was diminished the most.

A similar result was reported by Řezanka et al. (2011). The nitrogen depleted culture of *T. minutus* produced 21.9% EPA, which was less than the control, which achieved 35.8% EPA of total fatty acids.

T. sp. LCR-Awa9/2 that was grown in a phosphate depleted culture produced 28% fatty acid, higher than the control medium. Unlike the study published by Řezanka et al. (2011), EPA production under phosphorous starvation was the lowest compared to the control and nitrogen limited cultures. *T. minutus* produced 39.8% EPA under phosphorous starvation while the control had 35.8% EPA of total fatty acid (Řezanka et al., 2011). In this study, the phosphorous starvation culture of *T.* sp. LCR-Awa9/2 produced more myristic acid and elaidic acid than EPA. Compared to the control, the phosphate depleted culture produced slightly more palmitoleic acid, DGLA, myristoleic acid, and α-linoleic acid.

Between 8 and 14 days of cultivation, the culture accumulated more elaidic acid, linoleic acid, and palmitoleic acid. Myristic acid, myristoleic acid, α-linoleic acid, arachidic acid, and arachidonic acid proportions were slightly increased. The other fatty acids were reduced, particularly EPA.

Another experiment was run to confirm the results of Experiment 22. As shown in Figure 53, the growth of each condition was similar to Experiment 22. The nitrogen and phosphate depleted cultures growth were slower after day 5. In the final day of cultivation, both N-depleted and P-depleted culture could only produce 1.4×10^7 cells/mL. The dry biomass weight were 1.98±0.13 and 1.89±0.34 g/L, respectively (Figure 54). The consistency between Experiment 22 and 23 shown by Figures 50, 51, and 53 is very pleasing. Similar to Experiment 17.2, N-depleted culture recovered quickly after more nitrogen was added. The ½N culture that was supplemented with more N on day 10 produced 3.1×10^7 cells/mL and 2.8±0.3 g/L dry biomass. The biomass was 1.4 times higher than ½N culture.

The control (BBMA) medium gave 8.1×10^7 cells/mL cultures with a dry biomass of 4.71±0.12 g/L. The BBMA medium was also grown in other flasks until day 12 before it was transferred into a complete starvation environment. The culture produced 8.8×10^7 cells/mL and 5.9±0.5 g/L dry biomass. Even though the culture was transferred into water, it continued to grow and even produced more biomass than the other cultures. There were two factors that could cause the biomass growth. First, there was no light limitation. The complete starvation culture continued the cultivation process until day 17 where the other cultures were harvested in day 14. This means fewer flask in the incubator resulting in more light for the two remaining flasks. The second was CO_2 supply. The CO_2 was continued to be sparged into the flasks so even though the nutrients were removed, the cell can still utilise carbon to produce biomass.

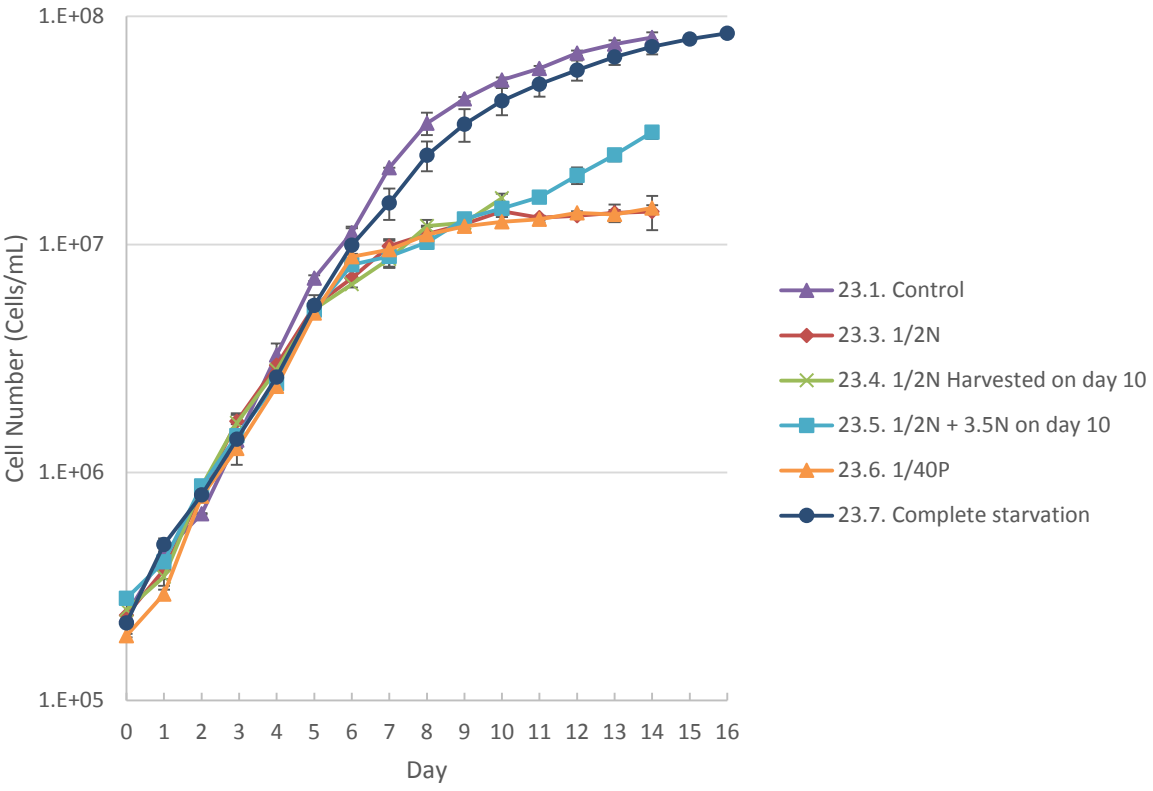

Figure 53. The effect of nutrient starvations to *T.* sp. LCR-Awa9/2 growth (repeated experiment).

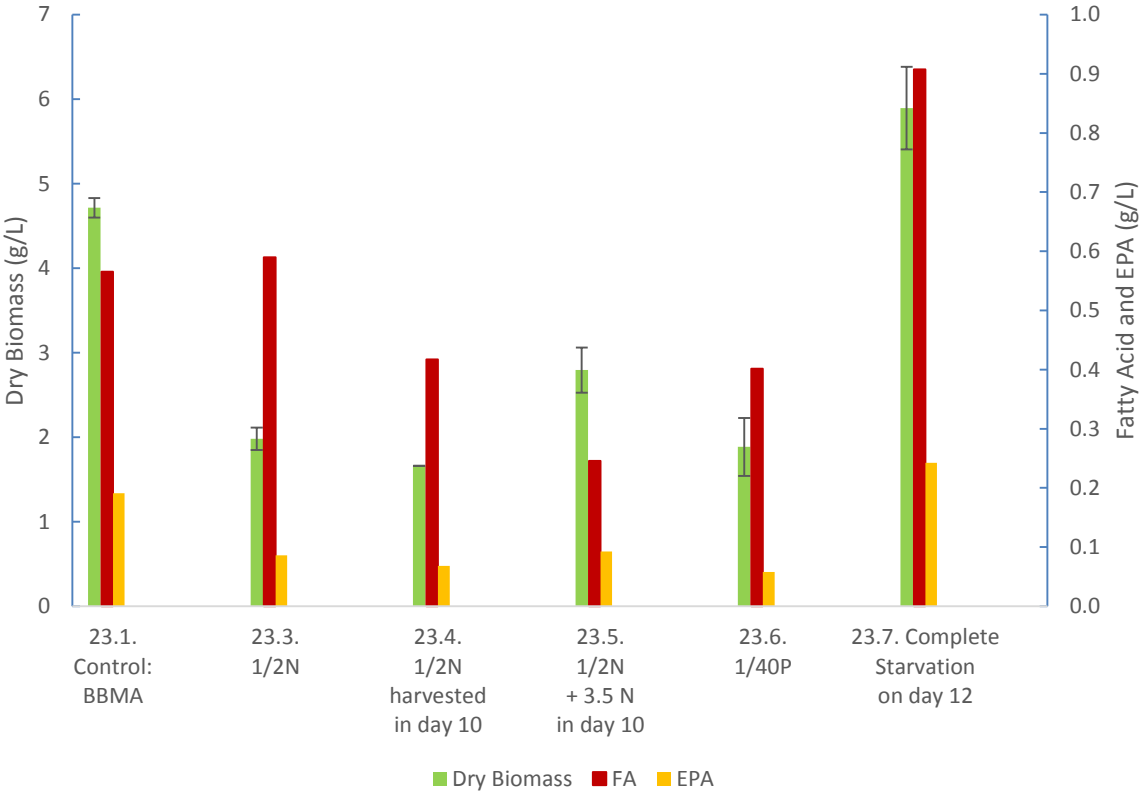

Figure 54. The effect of N, P, and complete starvation to biomass, fatty acid, and EPA production of *T.* sp. LCR-Awa9/2 (repeated experiment).

The fatty acid profile of nitrogen, phosphate, and complete starvation from Experiment 23 is given in Table 10 and 11. The fatty acid profile of the control, ½N, and $^{1}/_{40}$ cultures were consistent with Experiment 22. The ½N culture that was supplemented by more nitrogen on day 10 stimulated two times higher g EPA/100 g of total FA in four days. However, the FA dropped from 25.1% to 8.8% of DM. The EPA on day 10 in ½N culture was 16.4 g EPA/100 g of total FA and it increased into 37.59 g EPA/100 g of total FA in day 14 after 0.01 M $NaNO_3$ was added to the culture on day 10. There were some reductions in FA profile during the recovery period from nitrogen starvation. The biggest reductions was in elaidic acid, followed by behenic acid, palmitoleic acid, myristic acid, stearic acid, linoleic acid, arachidic acid, vaccenic acid, and DGLA.

The total FA increased from day 8 until day 14 in ½N culture. The FA profile change from day 8, day 10, and day 14 can be seen in Figure 55. The reduction of EPA (20:5n-3) happened during the nitrogen starvation period. The nitrogen starvation culture stimulated more elaidic acid production than EPA.

Figure 55. The fatty acid profile of nitrogen starvation cultures on days 8, 10, and 14.

The complete starvation culture produced 15% FA in DM, higher than the control medium. Elaidic acid production increased during the complete starvation. There were some reductions in FA profile during the complete starvation which were EPA (the highest reduction), myristoleic acid, 16:02, vaccenic acid, α-linolenic acid, and arachidic acid.

Table 10. The effect of N and P starvation on fatty acid profile in *T. sp.* LCR-Awa9/2 (Exp.23).

Fatty Acid g/100g Total FA	**23.1. Control: BBMA**	**23.3. ½N**	**23.4. ½N harvested in day 10**	**23.5. ½N + 3.5 N in day 10**	**23.6. $^{1}/_{40}$P**	**23.7. Complete Starvation on day 12**
Myristic acid (14:0)	16.50	21.58	19.91	15.95	26.09	17.47
Myristoleic acid (14:1n-5)	0.63	0.27	0.27	1.14	0.60	0.43
Palmitic acid (16:0)	7.62	6.66	6.78	7.45	4.69	8.39
Palmitoleic acid (16:1n-7)	10.10	12.56	12.51	7.84	12.98	10.62
16:02	0.48	0.06	0.08	0.42	0.26	0.33
Stearic acid (18:0)	1.23	1.69	1.70	0.39	1.10	1.86
Elaidic acid (18:1n-9)	3.64	20.53	19.95	3.30	13.36	6.74
Vaccenic acid (18:1n-7)	0.18	0.25	0.32	0.15	0.16	0.16
Linoleic acid (18:2n-6)	6.69	7.42	6.62	5.84	7.53	7.39
α-Linolenic acid (18:3n-3)	2.17	0.71	0.79	4.23	2.46	1.73
Arachidic acid (20:0)	0.54	0.39	0.41	0.14	0.30	0.65
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.55	0.66	0.77	0.68	2.63	0.79
Arachidonic acid (20:4n-6)	3.44	1.58	1.74	4.79	2.97	3.12
Eicosapentaenoic acid (**20:5n-3**)	33.75	14.62	16.40	37.59	14.45	26.71
Behenic acid (22:0)	7.22	8.41	9.04	3.30	5.90	9.22
Total fatty acids, %	**12.00**	**29.80**	**25.10**	**8.80**	**21.30**	**15.40**

Table 11. The fatty acid profiles fraction of total fatty acid (Exp.23).

FA g/100 g DM	**23.1. Control: BBMA**	**23.3. ½N**	**23.4. ½N harvested in day 10**	**23.5. ½N + 3.5 N in day 10**	**23.6. $^{1}/_{40}$P**	**23.7. Complete Starvation on day 12**
Myristic acid (14:0)	1.98	6.43	5.00	1.40	5.56	2.69
Myristoleic acid (14:1n-5)	0.08	0.08	0.07	0.10	0.13	0.07
Palmitic acid (16:0)	0.91	1.98	1.70	0.66	1.00	1.29
Palmitoleic acid (16:1n-7)	1.21	3.74	3.14	0.69	2.76	1.64
16:02	0.06	0.02	0.02	0.04	0.06	0.05
Stearic acid (18:0)	0.15	0.50	0.43	0.03	0.23	0.29
Elaidic acid (18:1n-9)	0.44	6.12	5.01	0.29	2.85	1.04
Vaccenic acid (18:1n-7)	0.02	0.07	0.08	0.01	0.03	0.02
Linoleic acid (18:2n-6)	0.80	2.21	1.66	0.51	1.60	1.14
α-Linolenic acid (18:3n-3)	0.26	0.21	0.20	0.37	0.52	0.27
Arachidic acid (20:0)	0.07	0.12	0.10	0.01	0.06	0.10
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.07	0.20	0.19	0.06	0.56	0.12
Arachidonic acid (20:4n-6)	0.41	0.47	0.44	0.42	0.63	0.48
Eicosapentaenoic acid (**20:5n-3**)	4.05	4.36	4.12	3.31	3.08	4.11
Behenic acid (22:0)	0.87	2.51	2.27	0.29	1.26	1.42
FA g/L	**0.57**	**0.59**	**0.42**	**0.25**	**0.40**	**0.91**
EPA g/L	**0.19**	**0.09**	**0.07**	**0.09**	**0.06**	**0.24**
g EPA/g dry biomass	**0.04**	**0.04**	**0.04**	**0.03**	**0.03**	**0.04**
Dry Mass (g/L)	**4.71**	**1.98**	**1.66**	**2.79**	**1.89**	**5.89**

6.4. Dynamic Model of Microalga *T.* sp. LCR-Awa9/2 Growth in a Flask

The experimental data of this thesis were used to develop a model by Weaver (2017). This simple model seemed to be effective to predict the growth of the alga, the nitrogen and phosphorous needs, and the light limitation. In the phosphorous starvation experiment, $^{1}/_{40}$P concentration was chosen by this model to give similar growth to that predicted for ½N.

In this model, some assumptions were made: the flasks are well mixed; the light intensity is the same for each flask (all the light that entered the flask went into the medium); there are no inhibiting products; growth is limited by nitrate, phosphate and light; other nutrients are in excess; Monod terms for growth rates were used; and the parameters are constant.

As explained in Pirt (1975), during a considerably small time interval (*dt*), the biomass (*dN*) is expected to increase to be proportional to the amount (*N*) of biomass and time interval.

$dN = \mu N.dt$

hence,

$$\frac{dN}{dt} = \mu N \quad (1)$$

The rate consumption of a substrate in a culture at a period of time is given by

$$\frac{dS}{dt} = -\mu N \mathrm{Y} \quad (7)$$

where Y is the consumption of a substrate per cell (g nutrient/g biomass). Hence the nitrogen consumption rate is

$$\frac{dS_N}{dt} = -\mu N Y_N$$

and the phosphorous consumption rate is

$$\frac{dS_P}{dt} = -\mu N Y_P m$$

The relation of the alga growth rate to substrate concentration will be

$$\mu = \mu_{max} \frac{S_N}{K_N + S_N} \frac{S_P}{K_S + S_P} \frac{I_{cell}}{K_I + I_{cell}} \quad (8)$$

where light per cell is

$$I_{cell} = \frac{I \cdot A}{N \cdot V} \quad (9)$$

Light per cell was used because of the turbulent flow in the shake flasks which enabled all cells to get a share of the light.

Table 12. Variables

Variables	Symbol	Unit
Independent:		
Time	t	s
Dependent:		
Growth rate	μ	s^{-1}
Number of cells	N	cells/mL
Initial number of cells	$N(t_0)$	cells/mL
Nitrogen substrate as NO_3^-	S_N	g/mL
Initial nitrogen substrate as NO_3^-	$S_N(t_0)$	g/mL
Phosphate substrate as PO_4^{3-}	S_P	g/mL
Initial phosphate substrate as PO_4^{3-}	$S_P(t_0)$	g/mL
Light flow per cell	I_{cell}	mol photon/cell.s or photon/cell.s

The modelling was performed by Weaver (2017) and Ken Morison using Matlab and Solver Excel following known parameters in Table 13 to find the unknown parameters: maximum growth rate, nutrients consumption rate, and Monod constants. The fitted simulation data was given in Figure 56 – 61. The value of unknown parameters is given in Table 14.

Table 13. Known parameter values

Known parameters	Symbol	Value	Unit
Light flux	I	268 (medium) 675 (high)*	µmol $m^{-2}s^{-1}$
Area of flask for light penetration	A	0.017	m^2
Volume of medium	V	100	mL
Cell mass	m	6×10^{-11}	g

*) The high light intensity based on Chapter III data was 530.6 µmol $m^{-2}s^{-1}$, 2.1× higher than the medium light intensity. The Matlab fitted this value into 2.7× higher than the medium light intensity.

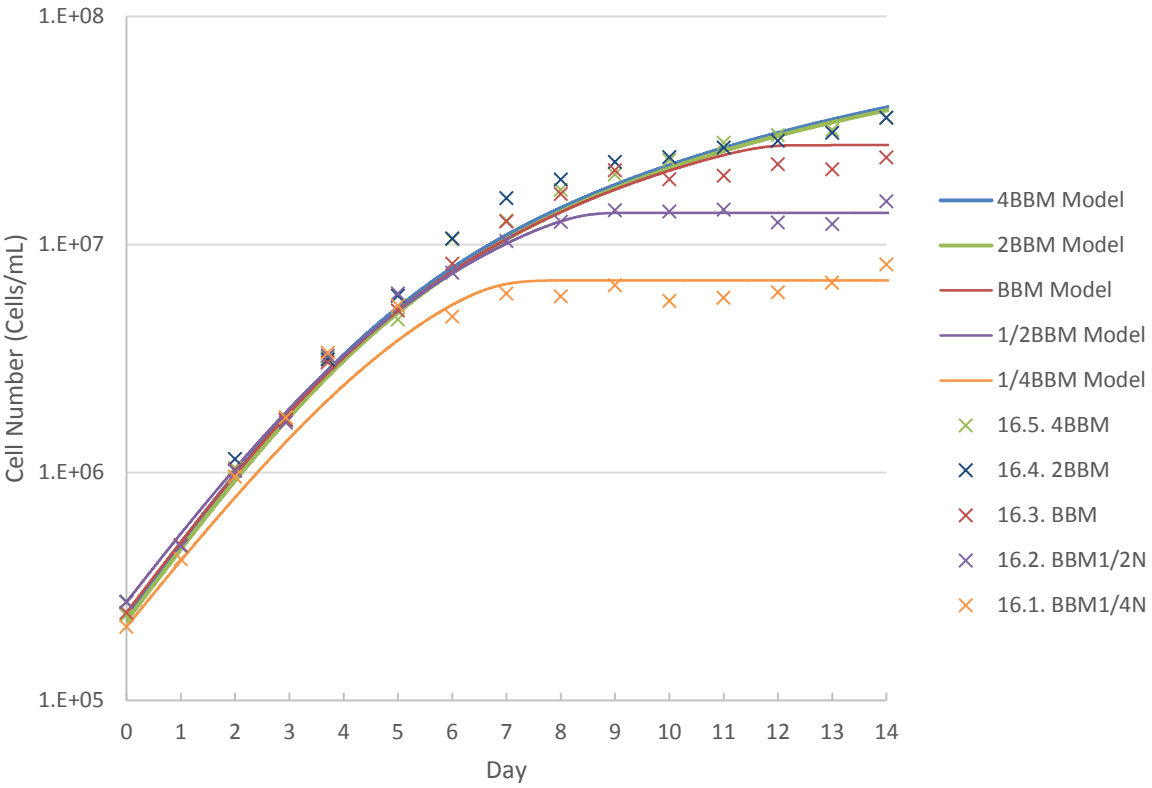

Figure 56. Fitted simulated data to experimental data from Experiment 16 (medium light intensity).

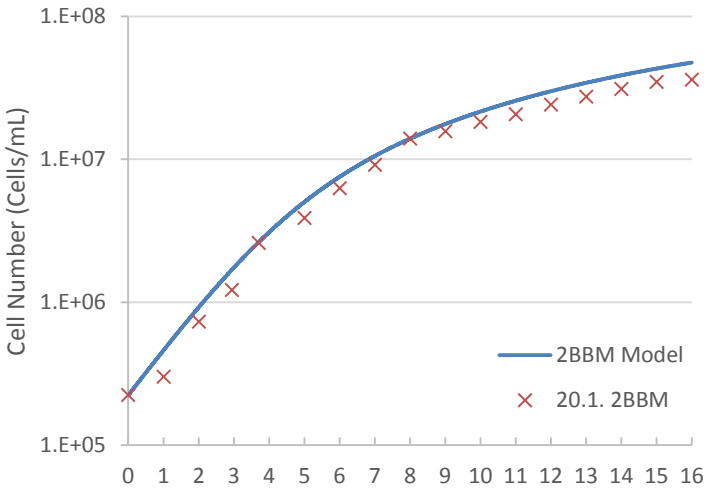

Figure 57. Fitted simulated data to experimental data from 2BBM, Experiment 20 (medium light intensity).

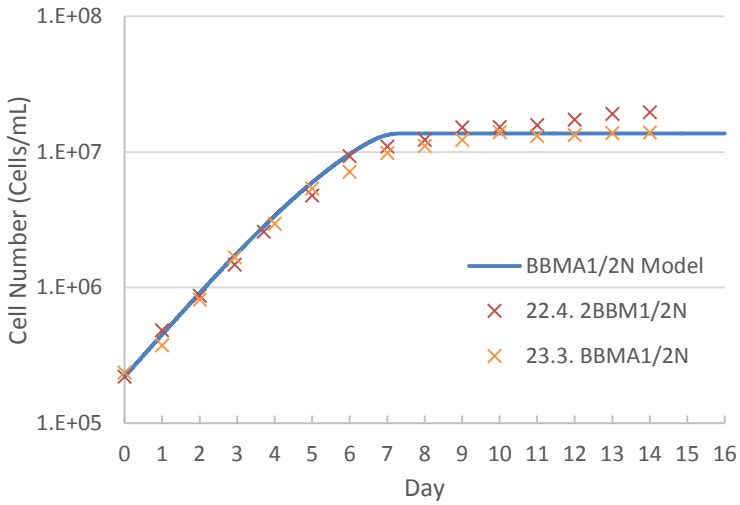

Figure 58. Fitted simulated data to experimental data from N starvation, Experiment 22 and 23 (high light intensity).

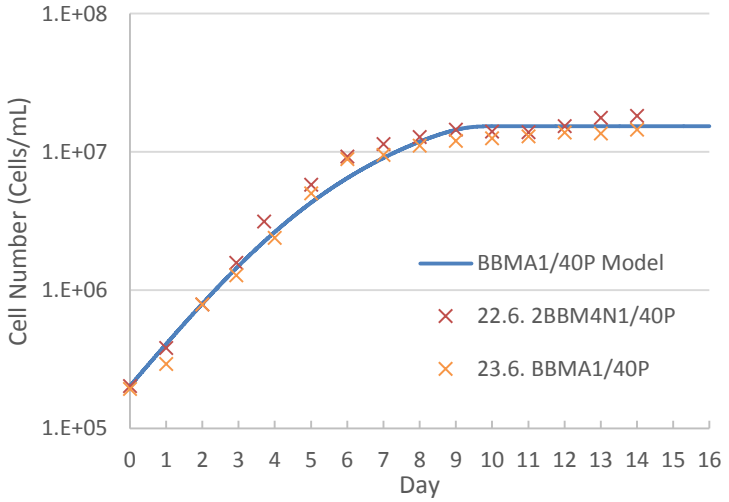

Figure 59. Fitted simulated data to experimental data from P starvation, Experiment 22 and 23 (high light intensity).

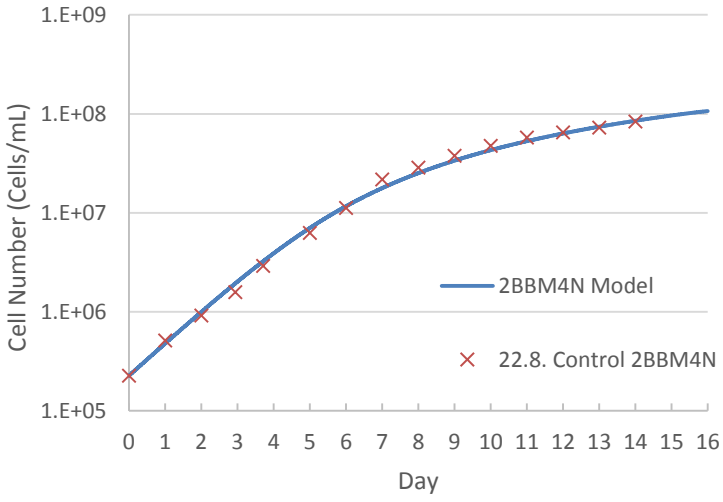

Figure 60. Fitted simulated data to experimental data from 2BBM4N, Experiment 22 (high light intensity).

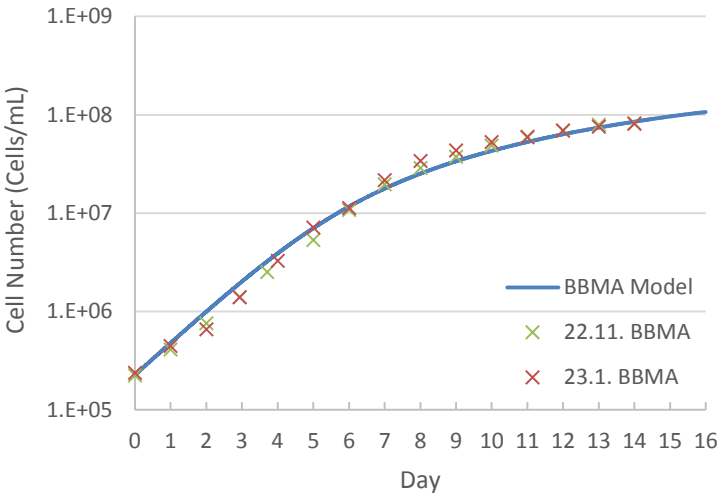

Figure 61. Fitted simulated data to experimental data from BBMA, Experiment 22 and 23 (high light intensity).

Table 14. Unknown parameters simulated by Solver

Unknown parameters	Symbol	Value	Unit
Maximum growth rate	μ_{max}	8.93×10^{-6}	s^{-1}
NO_3^- consumption ratio	Y_N	0.1113	$g_{NO_3^-}/g_{biomass}$
PO_4^{3-} consumption ratio	Y_P	0.000431	$g_{PO_4^{3-}}/g_{biomass}$
Monod constant for NO_3^-	K_N	0.00000471	g/mL
Monod constant for PO_4^{3-}	K_P	0.000000185	g/mL
Monod constant for light	K_I	6.5×10^{-15} 3.93×10^{9}	mol/cell.s photon/cell.s

The fitted simulated data fit very well to the experimental data.b This model was used to predict the concentration of low phosphate to have similar growth with ½ nitrogen cultures. The model predicted that $^1/_{40}$P would be similar to ½N and the experimental results proved that they had similar growth curve (Figure 58 and 59). These showed that the model is effective to describe the population growth.

6.5. Conclusions

Nitrogen, phosphorous, and complete starvation stimulated fatty acid production, but not the EPA. The highest yield of EPA production was obtained by the control (Exp. 22) which was 0.21 g EPA/L culture or 0.046 g EPA/g dry biomass. The nitrogen and complete starvation stimulated more elaidic acid, while the phosphate starvation produced more elaidic acid and myristic acid (compared to the control). The consumption of nitrate was 0.1113 g/g biomass and phosphate 0.000431 g/g biomass.

Chapter VII

EVALUATION OF TRACE ELEMENTS IN THE CULTIVATION OF *Trachydiscus* sp. LCR-Awa9/2

7.1. Introduction

Micronutrients are necessary for microalgae growth. This chapter reports the experiment results about the role of micronutrients in *Trachydiscus* sp. LCR-Awa9/2 growth and the final prototype medium that can be used for ongoing research about the microalga.

7.2. Methods

The flasks used were 250 mL baffled flasks, contained 100 mL culture media. The initial cell density was 2×10^5 cells/mL. The cultivation process took place in the MaxQ™ 6000 incubator. The temperature was maintained at 25 °C. The culture was exposed to medium light intensity and shaken at 150 RPM. 3% CO_2 enriched air was supplied into the culture with a total mass flow rate for all flasks of 500 mL/min and a 0.2 µm filter was used to sterilize the gas.

The biomass was harvested by centrifugation at 2907 RCF for 15 minutes. The liquid was disposed then the samples were dried using freeze dryer.

7.2.1 Evaluation of trace elements in BBM for *Trachydiscus* sp. LCR-Awa9/2 growth

The first experiment was performed to observe the effect of each trace elements in Bold Basal medium. Eighteen flasks were prepared for an experiment with nine culture media in duplicate. Bold Basal medium in a quarter concentration with four times sodium nitrate concentration (¼BBM4N) was used as control. The nitrogen concentration was increased to four times concentration to prevent early nitrogen starvation so the micronutrient effect could be evaluated without nitrogen starvation inhibition. In its initial nitrogen concentration (0.003 M), nitrogen starvation began in day 8 while the cultivation run for 14 days (see Experiment 5). ¼BBM4N without any micronutrients was also prepared as one of media to be observed. Seven ¼BBM4N solutions were made, each lacking one of the chemicals, which were boric acid, EDTA, zinc sulphate, manganese chloride, molybdenum trioxide, copper sulphate, and cobalt nitrate.

The sample from the culture stock was centrifuged at 3000 RPM for 10 minutes and the supernatant was disposed. The pelleted microalga cells were washed once with ¼BBM4N without micronutrients and re-suspended in the same medium before it was transferred into a new culture. The culture was cultivated for 5 days in ¼ BBM4N without micronutrients, in the same condition as the experiment, and then used as inoculum to start the experiment. The experiment runs were:

19.1. ¼BBM4N
19.2. ¼BBM4N without micronutrients
19.3. ¼BBM4N without boron
19.4. ¼BBM4N without iron and EDTA
19.5. ¼BBM4N without zinc
19.6. ¼BBM4N without manganese
19.7. ¼BBM4N without molybdate
19.8. ¼BBM4N without copper
19.9. ¼BBM4N without cobalt

7.2.2 The effect of depleted vitamins and selenium culture medium for *T.* sp. LCR-Awa9/2 growth

The next experiment was carried out to observe the effect of other trace elements. The inoculum was grown in Bold Basal medium, and the control medium was Bold Basal medium with twice concentration of nitrogen (BBM2N). Three BBM2N culture medium were prepared, each was supplemented by vitamins (thiamine HCl 17.5µg, biotin 25 µg, vitamin B12 15µg -the vitamins concentration was the same as OHM recipe-), selenium dioxide (0.05µg), and selenous acid (0.058µg). Four culture media, each in duplicate, were prepared in 8 baffled flasks. The inoculum was grown in Bold Basal medium at the same conditions as the experiment. The experiment runs were:
20.1. BBM2N
20.9. BBM2N that was supplemented by vitamins
20.10. BBM2N that was supplemented by selenium dioxide
20.11. BBM2N that was supplemented by selenous acid

7.2.3 The effect of different sources of iron to *T.* sp. LCR-Awa9/2 growth

The next batch of experiments to observe the effect of different sources of iron was performed. Four culture media, each in duplicate, were prepared in 8 flasks. Bold Basal medium with double concentration of sodium nitrate was used as the control medium. Three BBM2N media were prepared with different source of iron, they were ferric chloride, ferric chloride combined with sodium citrate, and ferrous sulphate combined with disodium EDTA. The iron concentration in all culture media tested was 1.63×10^{-4} M. The experiment conditions were:
20.1. BBM2N that used Fe(III)Na EDTA as iron source and chelating agent
20.5. BBM2N that used $Fe(III)Cl_3$ and Na-Citrate as iron source and chelating agent
20.6. BBM2N that used $Fe(II)SO_4$ and Na_2EDTA as iron source and chelating agent
20.7. BBM2N that used $Fe(III)Cl_3$ as iron source

7.3. Result and Discussion

7.3.1 The evaluation of BBM's micronutrients in *T.* sp. LCR-Awa9/2 growth

The micronutrient experiment was performed to identify the nutrients essential for *Trachydiscus* sp. LCR-Awa9/2. The concentration of nitrogen was increased to four times concentration to ensure that the nitrogen was non-limiting. Two of the seven chemicals tested, molybdenum and copper, produced a biomass slightly higher than the control medium when

absent from the culture medium. In terms of cell density, culture media without molybdenum, copper and cobalt showed a higher cell density than the control medium on the final day of cultivation. The result showed that the microalga growth was not effective without any micronutrients. The culture without micronutrients showed the lowest biomass and cell density.

As shown in Figure 62, of the seven chemicals tested, the culture medium without manganese produced the least biomass and had the lowest cell density, but not as low as the medium with no micronutrients. It means manganese was not the only essential micronutrient. The culture medium without boron and EDTA also produced a lower biomass and cell density than the control medium, while the culture media without zinc had slightly less biomass and cell density than the control.

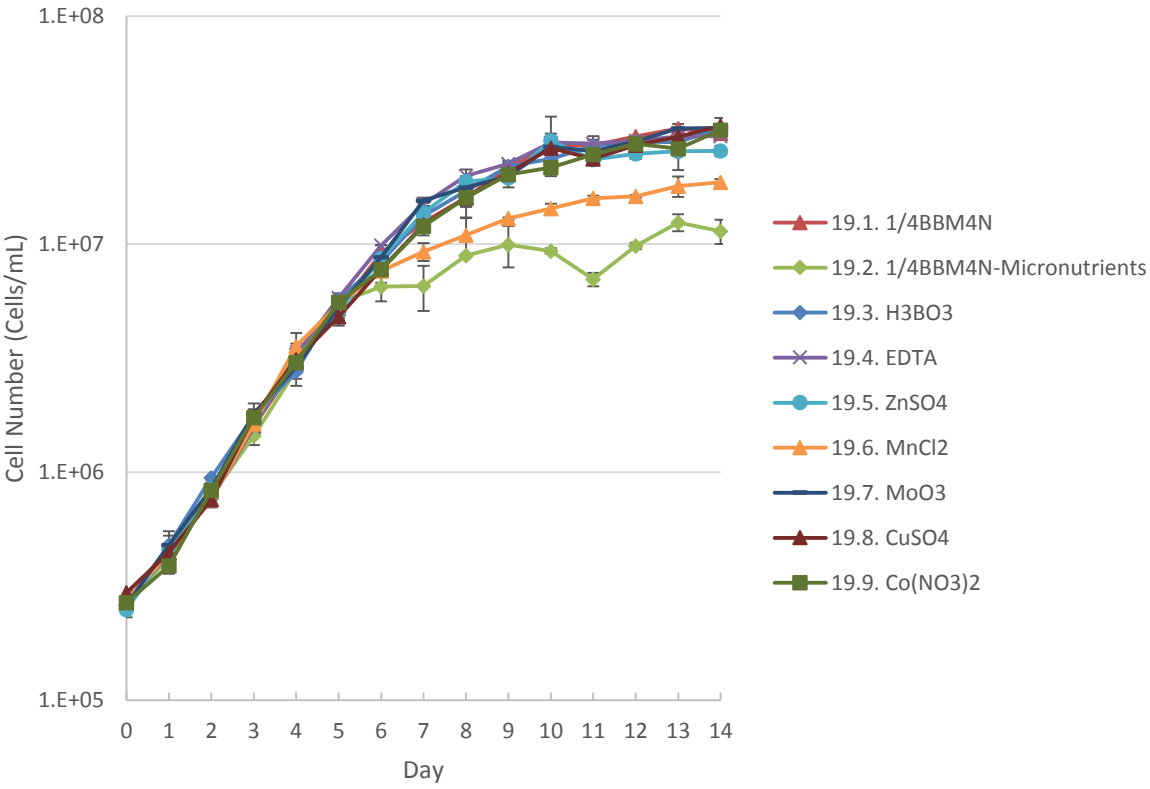

Figure 62. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in ¼ concentration of Bold Basal Media with four times N concentration and without the indicated nutrient.

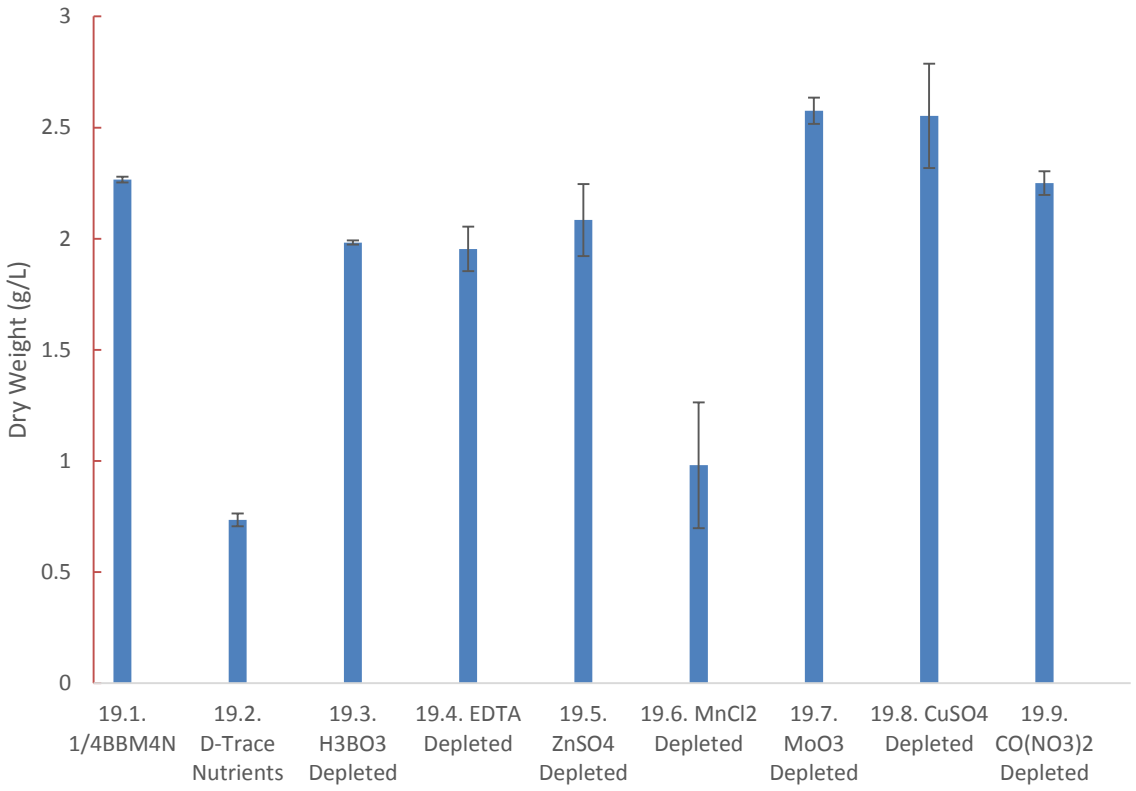

Figure 63. Biomass production performance of *Trachydiscus* sp. LCR-Awa9/2 in ¼ Bold Basal Media with four times N concentration without the indicated nutrient.

In order to simplify the culture medium, the results were used to select micronutrients that could be removed from Bold Basal medium.

Zinc and copper are popular and available in all media tested in Chapter IV (except zinc which is absent in OHM). In this experiment, on the final cultivation day, the culture media without zinc produced less biomass and cell density than the control medium. This indicates the importance of zinc in the growth of the alga. In contrast to zinc, copper had more biomass and cell density than the control on the final day of cultivation, but had gradual lower cell density than the control from the first day of cultivation to day 13. This result was in contrast with the result reported by Fábregas et al. (2000), in which zinc was demonstrated to be toxic as a higher cell density was obtained when it was absent. Copper was tested in four different concentrations (0, 0.012, 0.12, and 1.2 mg/L) and the optimum growth was obtained from the culture medium with 0.025 mg/L copper. This was lower than the range of copper discovered in previous studies (written in Chapter II). The concentration of zinc and copper in Bold Basal medium were 1.57 mg/L and 8.82 mg/L, higher than the value given by that previously reported experiments in Chapter II, where zinc concentration was between 0.25 – 2 mg/L, and copper between 0.2 – 0.5 mg/L.

Molybdenum is available in all culture media, which were tested in chapter IV. In this experiment, a culture medium without molybdate did not show a significant difference to the control medium. Molybdate concentration in Bold Basal medium was 0.71 mg/L.

Despite the unspecified role of boron in microalgae growth, almost all culture media that were tested in previously reported experiment used boric acid, with the exception of Optimum Haematococcus medium. *Trachydiscus* sp. LCR-Awa9/2 cultivation in OHM and BBM gave the best result in comparison with the other culture media. In this experiment, the culture medium without boron showed a slightly lower growth rate than the control medium. This result corresponded with Fabregas et al. (2000), indicated that boron might be toxic for the alga. Removing boron from the culture medium was then considered.

Cobalt is included in the formulation of all culture media tested in Chapter IV. In this experiment, during the exponential phase, culture media without cobalt showed higher cell density than the control. The number became lower than the control in the stationary phase; however, after harvesting, the culture showed a higher biomass than the control. The result confirmed that cobalt is less effective than zinc for carbonic anhydrase and it did not agree with Fábregas et al. (2000).

Removing cobalt from the culture medium was considered due to the result of Experiment 19. Experiment 22 was performed to confirm the previous result (Figure 64). The microalga was grown in BBM4N without cobalt. The inoculum was grown in BBM4N with limited cobalt (1.15×10^{-7} M). It was centrifuged and washed two times with BBM4N without cobalt before being transferred to ensure that none of the cobalt was carried into the new culture.

There was no significant difference between the tested culture medium and the control in terms of growth rate. The culture in 2BBM4N without cobalt produced 4.06 g/L dry biomass, higher than the control, which gained 3.82 g/L dry biomass. The experiment showed a result consistent with Experiment 19, namely that the culture could grow more (both in cell density and dry biomass) without cobalt.

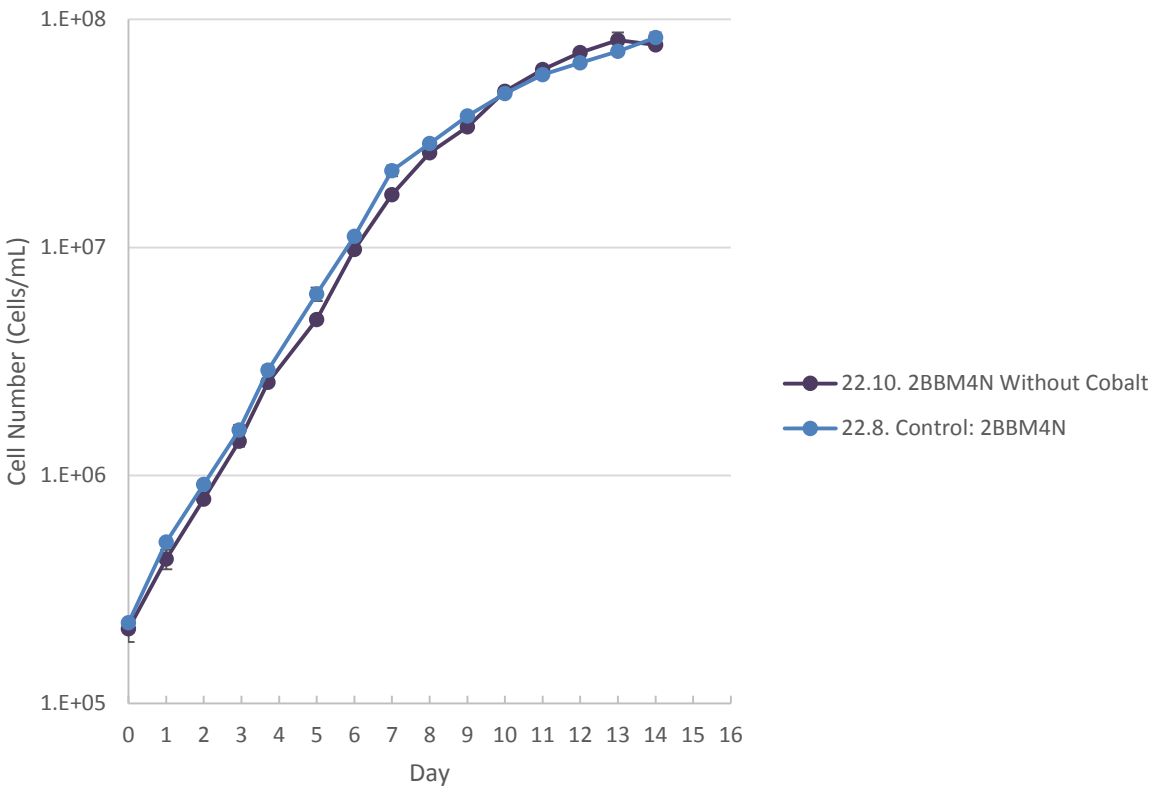

Figure 64. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 grown in 2BBM4N without cobalt.

7.3.2 The effect of depleted vitamins and selenium culture medium for *T.* sp. LCR-Awa9/2 growth

Another experiment was performed to observe the role of selenium and vitamin in microalgae cultivation (Figure 65). Between the eight culture media that were tested in the previous experiment, only OHM and MLA used selenium in their formula. OHM had 4.5×10^{-10} M of selenium dioxide, while MLA used 9.3×10^{-10} M of selenous acid. These concentrations were lower than the toxic level, which was 1µM (Araie & Shiraiwa, 2016). Araire & Shiraiwa (2016) reported that so far there were 31 microalgae species that required selenium for their growth. Fábregas et al. (2000) also discovered that *Haematococcus pluvialis* growth was improved by the presence of selenium. Selenium was required for Se-metabolism in selenoproteins and detoxified compounds.

The growth of microalga in bold basal medium with two times concentration of N supplemented by 4.5×10^{-10} M selenium was observed.

In this experiment, the culture medium that was supplemented by selenium dioxide showed only a small stimulative effect on growth, while selenous acid was less effective than selenium dioxide. It was not expected that selenium dioxide and selenous acid would give different results because when selenium dioxide dissolves in water, it forms selenous acid.

This result confirmed the first hypothesis that it was selenous acid that inhibited the growth of the microalga in MLA medium in the previous experiment. The culture had 6 days of lag phase

before it began to grow (Figure 32). In the preliminary experiment, MLA with selenous acid performed poorly but when the selenous acid was removed from the culture medium, the microalgae growth rate increased (Experiment 7).

Neither of the selenium cultures gave a significant rise in terms of cell number, but the selenium dioxide supplemented culture has slightly higher dry biomass than the control, 2.7±0.2 g/L (Figure 66).

The amount of vitamins added into the culture medium were 25, 17.5, and 15 µg/mL biotin, thiamine, and B_{12}, respectively. Unlike Fábregas et al. (2000), adding vitamins into the culture medium did not give significant improvement. The dry biomass was only 2.3±0.2 g/L, lower than the control which was 2.5±0.2 g/L. Adding vitamin was then not further considered due to the insignificant production improvement and the expensive price.

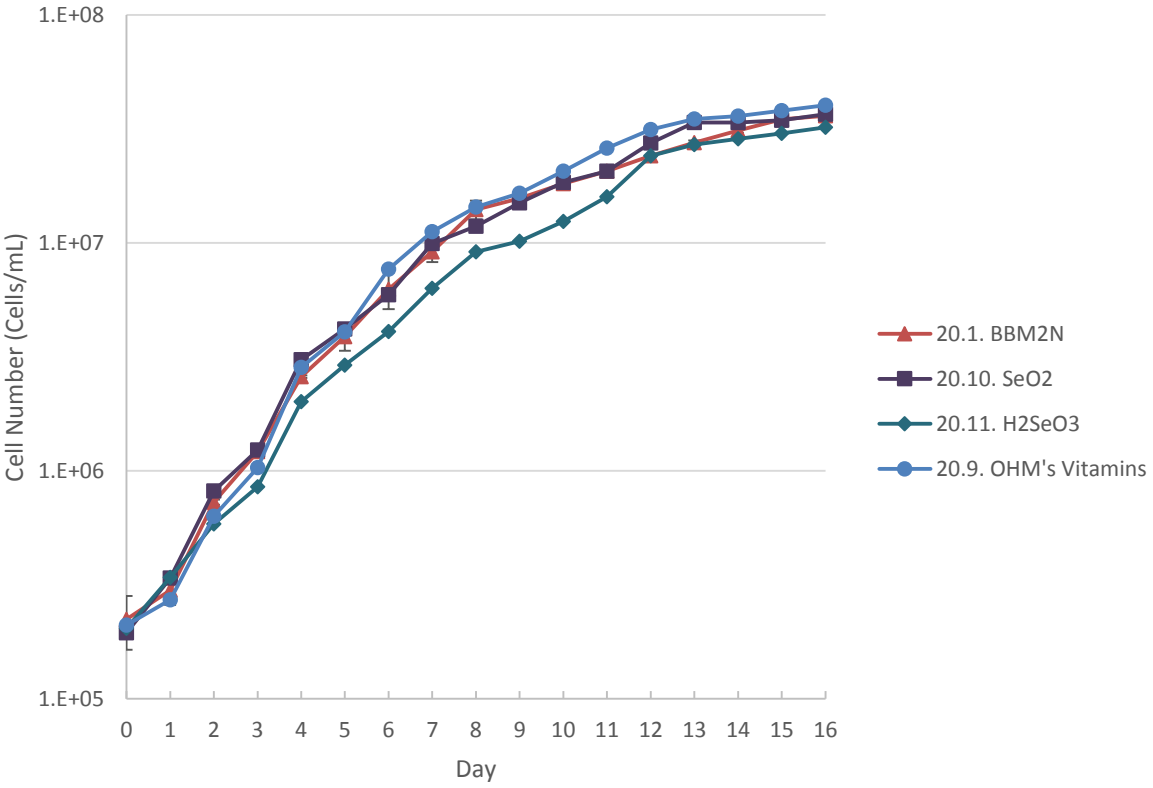

Figure 65. Growth curve of T. sp. LCR-Awa9/2 in 2BBM of sodium nitrate, supplemented with vitamin and different sources of selenium.

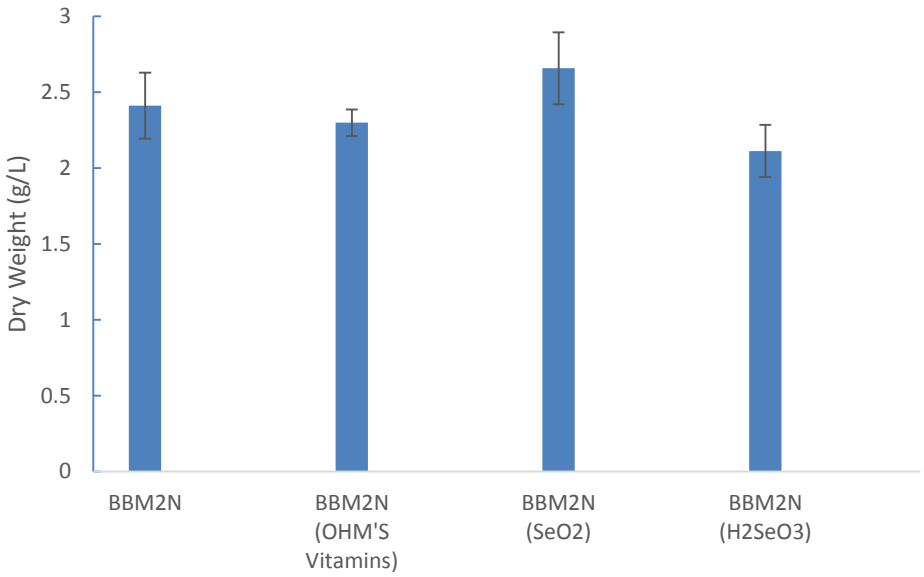

Figure 66. Dry mass from cultivation of T. sp. LCR-Awa9/2 in Bold Basal medium with double concentration of sodium nitrate, supplemented with vitamin and different sources of selenium.

7.3.3 The effect of different sources of iron to *T.* sp. LCR-Awa9/2 growth

Another experiment was done to study the effect of different sources of iron on the microalga growth. This experiment tested three different sources of iron: Fe-EDTA^{-} from Fe-Na-EDTA, Fe^{3+} from $FeCl_3$, and Fe(III)Cl_3 + Citrate, and Fe^{2+} from Fe(II)SO_4+Na_2EDTA (Exp.20) and Fe(II)SO_4 (Exp.19). The concentration of iron was 1.63×10^{-4} M, EDTA 1.63×10^{-4} M, and citrate 1.74×10^{-5} M (from OHM's citrate concentration).

The microalgal culture with Fe(II)SO_4 without EDTA showed a slightly lower growth rate than the control (1/4BBM, using Fe(III)Na EDTA, Experiment 19, Figure 67). The final cell density was 2.9×10^{7} cells/mL and 3×10^{7} cells/mL respectively. The Fe(II)SO_4 without EDTA culture produced 2.0±0.1 g/L biomass and the control produced 2.2±0.01 g/L (Figure 68).

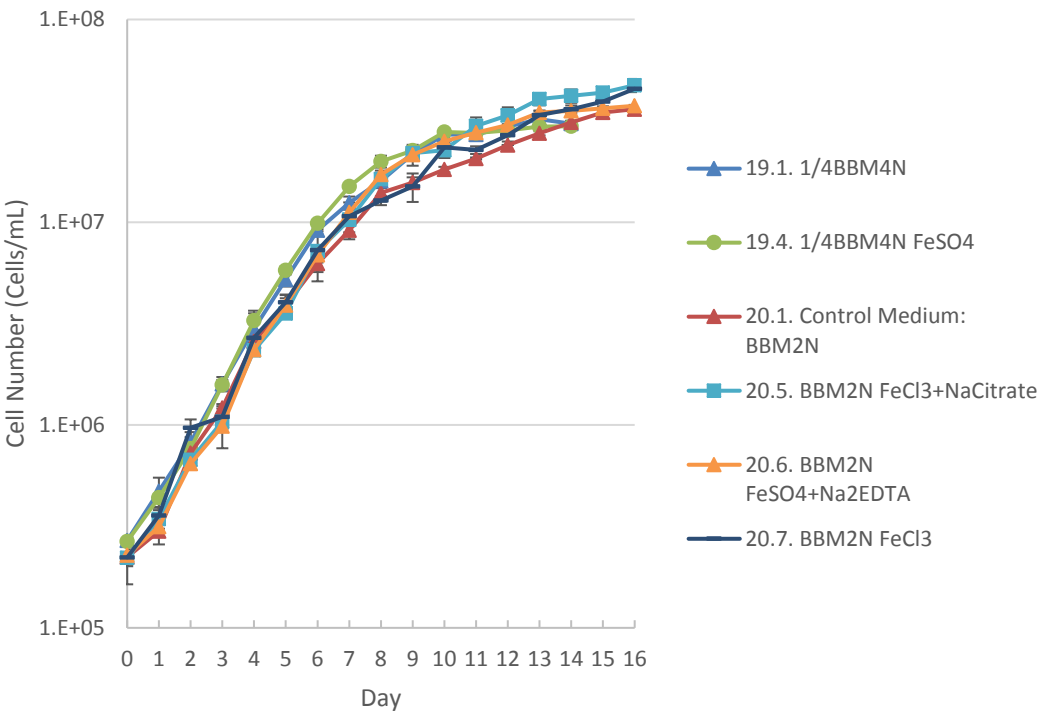

Figure 67. Growth curve of T. sp. LCR-Awa9/2 in Bold Basal medium with double concentration of sodium nitrate, using different sources of iron (Exp.20) and 1/4BBM (Exp.19).

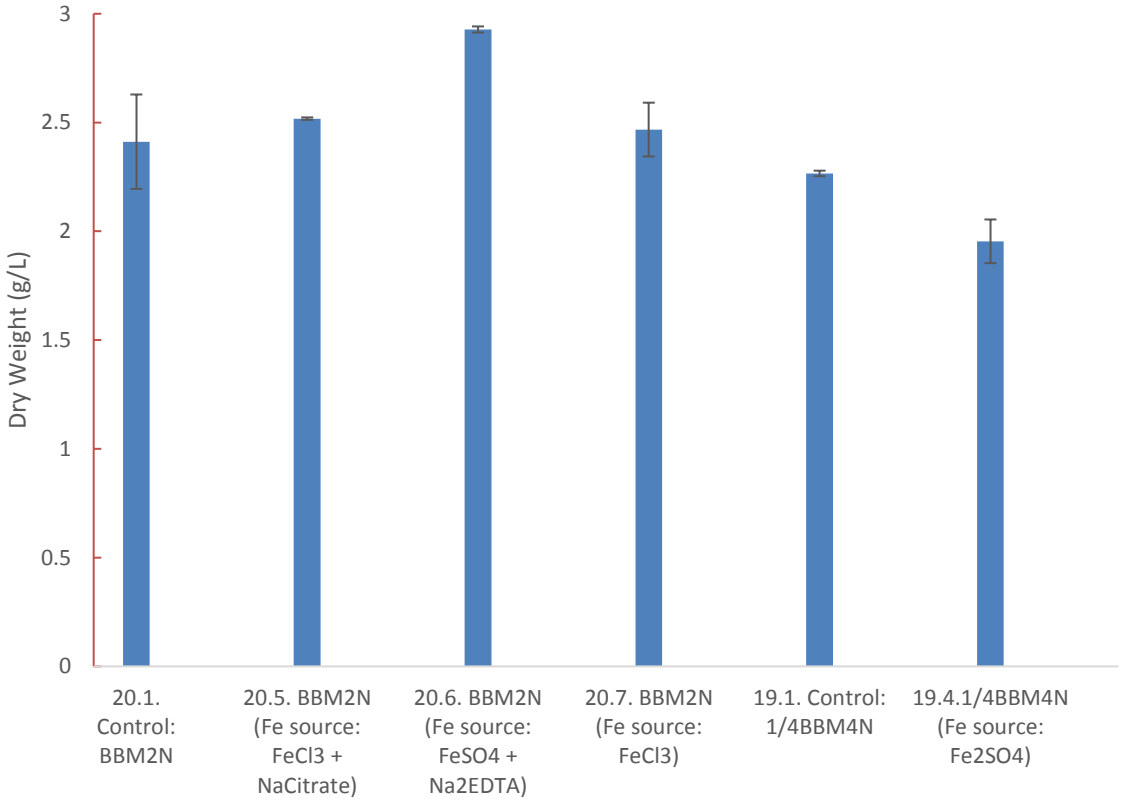

Figure 68. Dry weight of biomass from cultivation of T. sp. LCR-Awa9/2 in Bold Basal medium with double concentration of sodium nitrate, using different sources of iron (Exp.20) and 1/4BBM (Exp.19)

In Experiment 20, all modified culture media were higher than the control. In terms of cell density, the culture media that used Fe(III)Cl_3 as their iron source and Na-Citrate as the chelating agent were higher than the control (which used Fe(III)Na EDTA) and culture medium with Fe(II)SO_4 and Na_2EDTA. The highest cell density was up to 4.55×10^7 cells/mL. However, in terms of dry biomass, the culture medium with Fe(II)SO_4 and Na_2EDTA gained the highest mass, at 2.9±0.01 g/L. Liu et al. (2008) preferred Fe(III)Cl_3 as an iron source rather than Fe(II)SO_4, because the presence of chloride, which is useful in triggering the oxidative stress that will result in lipid accumulation. In BBM, which is the control medium in this experiment, the concentration of chloride does not change greatly if the iron source is changed to Fe(III)Cl_3, and it will therefore not be necessary to change Fe(II)SO_4 to Fe(III)Cl_3 if the consideration is based on chloride concentration.

This experiment resulted in the conclusion that *T.* sp. LCR-Awa9/2 was able to grow with either ferric or ferrous iron supplemented culture media, although the iron had different anions. There was no growth inhibition and all iron sources appeared to maintain the growth sufficiently. The growth of all culture media during the exponential phase was similar and some differences appeared in the stationary phase. Based on the final cell density and dry biomass, chelated iron compounds appeared to increase the cell growth a little, compared to culture media without EDTA or citrate (20.7 and 19.4). It was decided to continue using Fe(III)Na EDTA.

The ability to grow in culture media supplemented by different sources of iron is common among many microalgae species. *T.* sp. LCR-Awa9/2 showed promising growth in six different culture media with different iron sources. Bold Basal medium, ½SS medium, and Zehnder medium used Fe(III)Na EDTA, Blue Green 11 used ammonium ferric citrate and Na_2EDTA, Modified Bourrelly medium used Fe(III)Na EDTA and Fe(II)SO_4, Alexander Svetoslav used Fe(II)SO_4, and OHM used ammonium ferric citrate and Fe(III)Cl_3. The same strain also showed good growth in MLA medium, which used Fe(III)Cl_3 and Na_2EDTA (based on Mehrnoush Tangestani's unpublished experimental result).

Chelated iron (Fe(III)Na EDTA and Fe(III)EDTA) and ferrous (Fe(II)SO_4 with Na_2EDTA as the chelating agent) have been used for *Trachydiscus minutus* cultivation (Alexandrov et al., 2014; Cepák et al., 2014; Gigova et al., 2012; Řezanka et al., 2010). Similar to *T. minutus*, another EPA producer microalgae, *Nannochloropsis limnetica* could also utilize chelated iron and ferrous, and in particular ferric (Fe(III)Cl_3 and ammonium ferric citrate) (Freire et al., 2016; Krienitz & Wirth, 2006; Parupudi et al., 2016).

The freshwater microalgae *Chlorella vulgaris* and *Chlorella sorokiniana* were able to grow in culture media using Fe(III)Cl_3, ferric ammonium citrate, or chelated ferrous compounds (Liu et al., 2008; Wan et al., 2014). For *Dunaliella tertiolecta*, Fe(III)Cl_3 and Fe(III)EDTA was commonly used for cultivation (Chen et al., 2011; Rizwan et al., 2017).

7.4. Conclusions and Recommendations

Manganese deprived culture showed poor growth in compared to the other micronutrients. But the trace element deprived culture gave the poorest growth, means manganese was not the only limiting nutrients. Cobalt and boron were demonstrated to be slightly toxic to the culture, therefore they were removed from the culture medium.

In term of biomass productivity, there was no significant rise from supplemented the culture with selenium and vitamins. Using different sources of iron and chelating agent also did not show significant improvement.

Chapter VIII

EFFECT OF DIFFERENT LIGHT INTENSITIES AND WAVELENGTH IN THE GROWTH OF *Trachydiscus* sp. LCR-Awa9/2

8.1. Introduction

The densest culture reached from Experiment 1 until Experiment 20 was 4×10^7 cells/mL. Weaver (2017) did a dynamic model of the alga population growth and found that the cell number could reach 8×10^7 cells/mL if the light intensity was increased to high light intensity. The model showed that light limitation happened after day 5 due to the growing number of cells. This experiment was performed to test the effect of different light intensities and wavelength to *Trachydiscus* sp. LCR-Awa9/2 population growth.

8.2. Methods

The inoculum used to start the experiment was grown in high light intensity to avoid a lag phase (the culture stock was maintained in medium light while the cultures in this experiment were exposed to high light intensity). 20 flasks were prepared for the experiment with each set of conditions in duplicate. 2BBM was used as the control and basis medium. The nitrate concentration was increased to two times concentration to prevent early nitrogen starvation so the light effect could be evaluated without nitrogen starvation inhibition. In its initial N concentration (0.003 M), nitrogen starvation began in day 8 while the cultivation would be running for 14 days (see Chapter V). There were ten conditions observed which were:
21.1. Control medium (2BBM)
21.2. Culture that shrouded by white plastic resulting in 67% light entered the flask,
21.3. Culture that shrouded by white plastic until day 5, resulting in 45% light entered the flask,
21.4., 21.5, 21.6. Culture that shrouded by green, red, and blue cellophane
21.7., 21.8., 21.9. Culture that shrouded by green, red, and blue cellophane after cell density reached 3×10^7 cells/mL,
21.10. Culture that shrouded by white plastic resulting in medium light intensity after cell density reached 3×10^7 cells/mL.

Another experiment was performed to evaluate the effect of sudden light reduction to fatty acid profiles. 2BBM4N was used as the control medium. The flask was covered with white plastic to reduce 55% of the light after the culture cell number reached 3×10^7 cells/mL.

The biomass concentration (g/L) was determined by centrifugation at 10000 rpm of 100 ml of microalgal culture contained in two 50 mL falcon tubes for 10 minutes. The supernatant was discarded and the cellular pellet was dried to constant mass using a freeze dryer.

The Photosynthetically Active Radiation (PAR) of the cellophane cover was measured using an Apogee Instruments Quantum sensor. The numbers were then converted into µmol

photons/m²s based on measurement in Chapter III (part *Light Source*). The data was given in Table 20.

Table 15. Light intensities of the cellophane covered flasks.

Light Intensity	**Filter Colour**	**Intensity** (µmol photons/m²s)	**The Light Intensity Reduction**
Low	Green	6.5	56%
	Red	6.5	56%
	Blue	4.9	67%
	2/3White	9.8	33%
	1/2White	6.5	56%
Medium	Green	110.6	56%
	Red	115.8	54%
	Blue	82.5	67%
	2/3White	170.2	32%
	1/2White	110.6	56%
High	Green	229.2	**57%**
	Red	245.0	**54%**
	Blue	179.8	**66%**
	2/3White	356.1	**33%**
	1/2White	239.7	**55%**

8.3. Results and Discussions

The light intensity measurements given in Chapter III show significant variations with positions and hence the absolute intensities are uncertain. However, the relative amount of light between medium and high setting and with filters was much more consistent. The measurements do not directly give the amount of light entering the culture and this will be affected by position and reflections of flasks. The difficulties here raise uncertainties about absolute light measurements reported by others (Table 1, Section 2.2.2)

The result of this experiment supported the hypothesis that light was the inhibiting factor in the previous experiments, which did not achieve more than 4×10^7 cells/mL. In this experiment, the control medium could produce 6.5×10^7 cells/mL. The number was expected to exceed 7×10^7 cells/mL if four times concentration of nitrogen was used in 2BBM as the nitrogen starvation seemed to appear on day 15. The colour change from dark green to pale green was a clear indication of N starvation (Figure 69).

Figure 69. The left tube was the control medium (6.5×10^7 cells/mL), the right tube was the culture that was shrouded by blue cellophane (2.6×10^7 cells/mL). The control medium was in nitrogen starvation phase after reached 5.5×10^7 cells/mL.

All cultures showed similar growth rates up to day 3 (Figures 70, 71, and 72). Starting from day 4, there were variations. The shrouded culture began to growth slower than the control medium. The culture with blue filters gave the lowest cell density, from day 4 to the day of harvest.

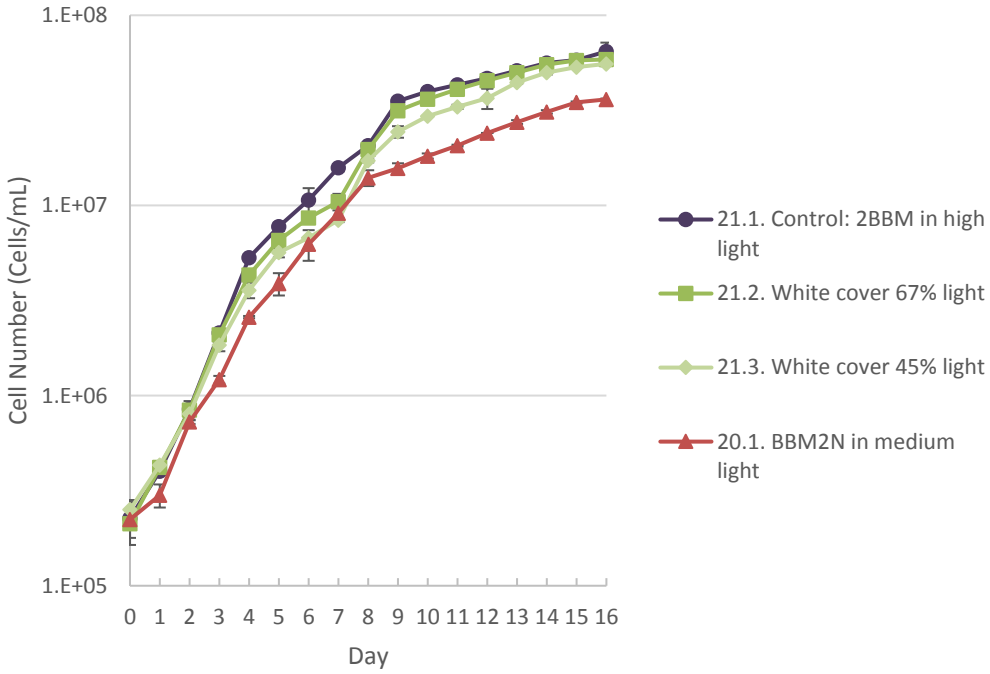

Figure 70. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 in different light intensities. The white covers were removed on day 5.

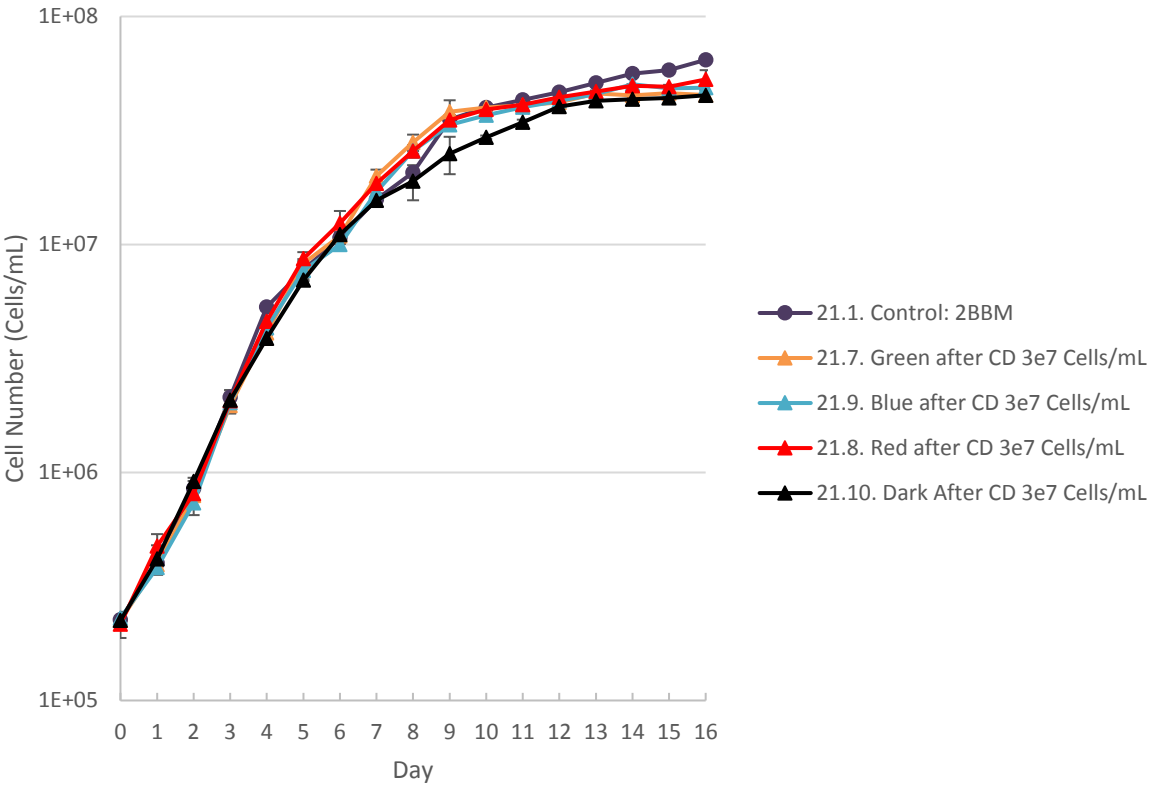

Figure 71. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 in different light intensities and colours after the culture reached 3×10^7 cells/mL.

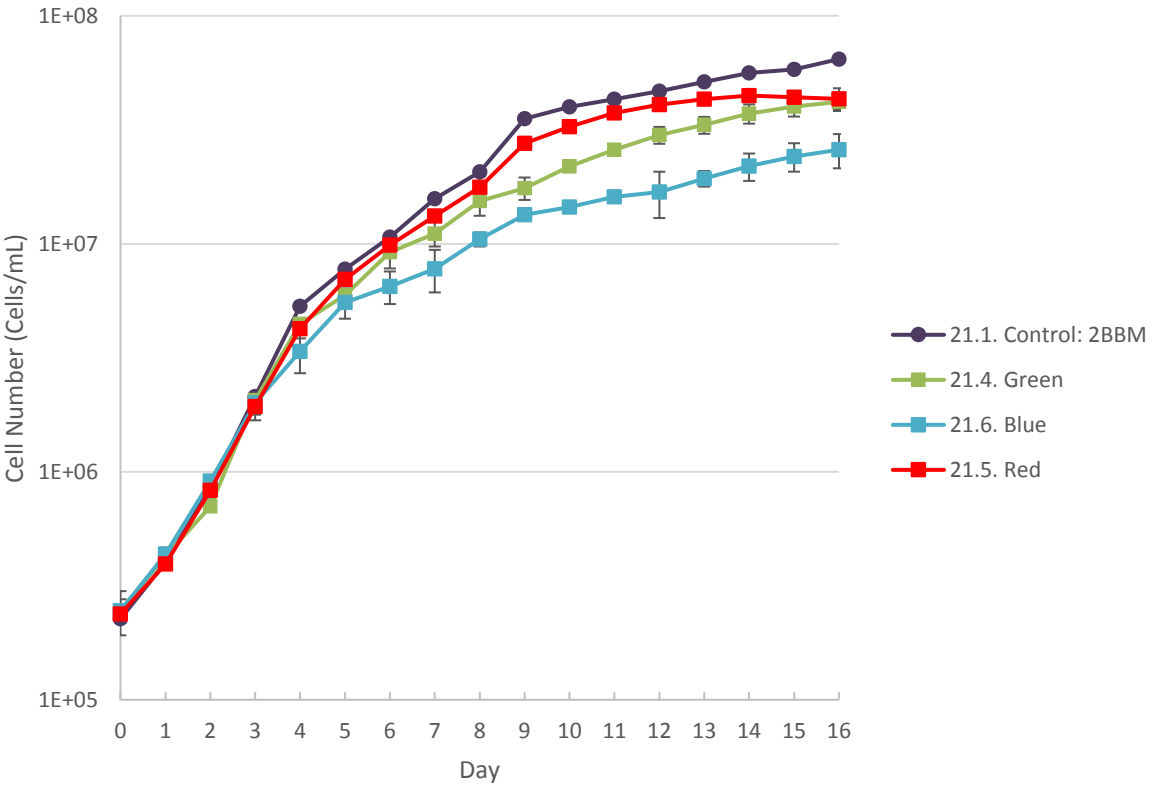

Figure 72. Growth curve of *Trachydiscus* sp. LCR-Awa9/2 in different light intensities and colours.

The cultures with 67% light and 45% light growth were slower on days 4 and 5, but after the filters were removed on day 5, the growth recovered and finally reached 5×10^7 cells/mL. This was still lower than the control on the harvest day due to the flasks' position and shade from flasks with colour filters. As described in Chapter III, different positions in the incubator had different light intensity. The back positions got more exposure to light, so culture that sit in the back always have higher growth than the one in front. Therefore, in each experiment, the flask positions were randomly moved every day to avoid the uneven distribution of light.

The highest light intensity in this study was measured to be 431-530 $\mu molm^{-2}s^{-1}$. The culture produced 4.08±0.01 g/L biomass. In a similar range of intensity, *T. minutus* produced 11.9 and 12.28 g/L biomass (Cepák et al., 2014; Řezanka et al., 2010), which could not be compared to *T.* sp. LCR-Awa9/2 on account of different experimental conditions. The culture started with a very dense inoculum, up to 4.2×10^7 cells/mL. *T.* sp. LCR-Awa9/2 culture was started with 2×10^5 cells/mL and when the culture reached 4×10^7 cells/mL, the liquid appeared very dense.

In the medium range of light intensity, 200-250 $\mu molm^{-2}s^{-1}$, *T. sp* LCR-Awa9/2 was only able to produce 2.4 g/L dry biomass or 3.6×10^7 cells/mL culture (Figure 73). A similar result was shown by some studies about *T. minutus* (Alexandrov et al., 2014; Gigova & Ivanova, 2015; Gigova et al., 2012; Řezanka et al., 2011). The biomass productions were lower than cultivation at higher light intensity.

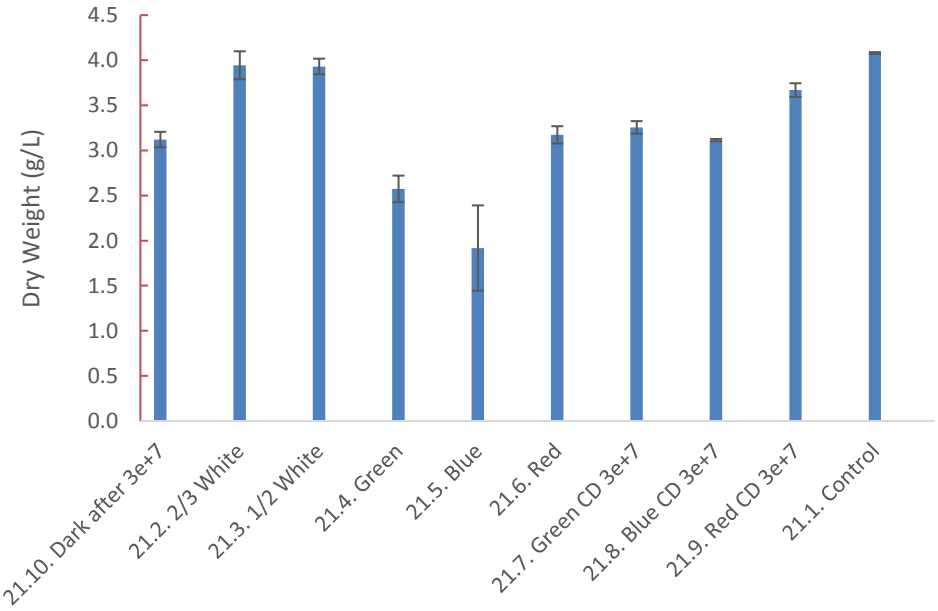

Figure 73. Biomass production of *Trachydiscus* sp. LCR-Awa9/2 in different light intensities and wave lengths.

The effect of covering the flask with cellophane can only be related to the light intensity, not the wavelength since the wavelength measurement for the cellophane has not been done. The cellophane blue reduced 66% of the light intensity, followed by red and green, 54% and 57% light reduction respectively. The result agreed with the hypothesis that the culture required more light. The lower the light intensity, the lower the growth. There was no significant change when the cultures were covered with the cellophane and aluminium foil (to create dark environment)

after the cell number reached 3×10^7 cells/mL. It was because the culture was in stationary phase and was already light limited due to the dense culture.

This experiment provided useful data for the model (using equations in Chapter 6) which had indicated limitation of growth due to insufficient light (Figure 74). When the light intensity was increased, the biomass increased was up to 1.5 g/L (the best production in Experiment 20 was only 2.5 g/L).

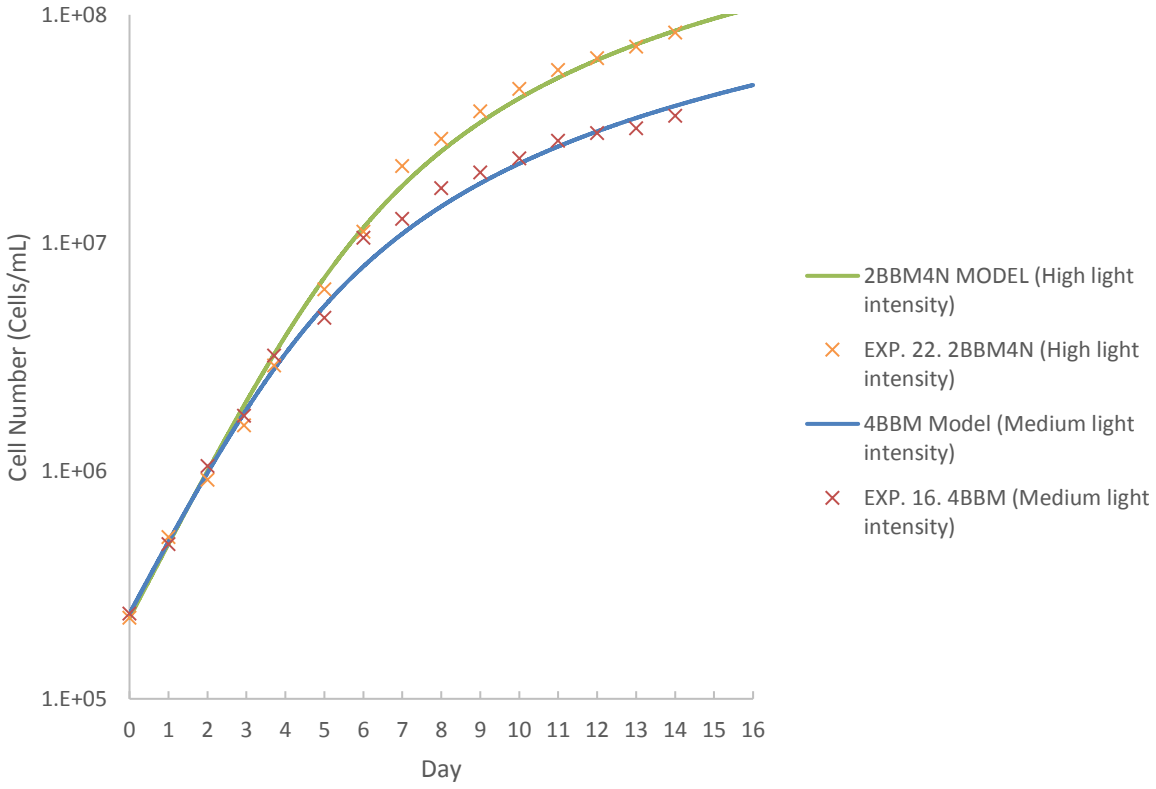

Figure 74. Fitted simulation data to experimental data from experiments with medium and high light intensity.

In Experiment 22, the 2BBM4N culture (22.2) was exposed to sudden light reduction after the cell number reached 3×10^7 cells/mL which happened on day 9. The culture produced 2.8 g/L dry biomass which was less than the control. The fatty acid profile is given in Figure 75. The total fatty acid of 22.2 culture was 10.8% dry weight biomass. It was lower than the control (without light reduction) that gained 13.7% FA in DM. Most of the fatty acids were lower than the control, except EPA which reached 37.67% of total fatty acid.

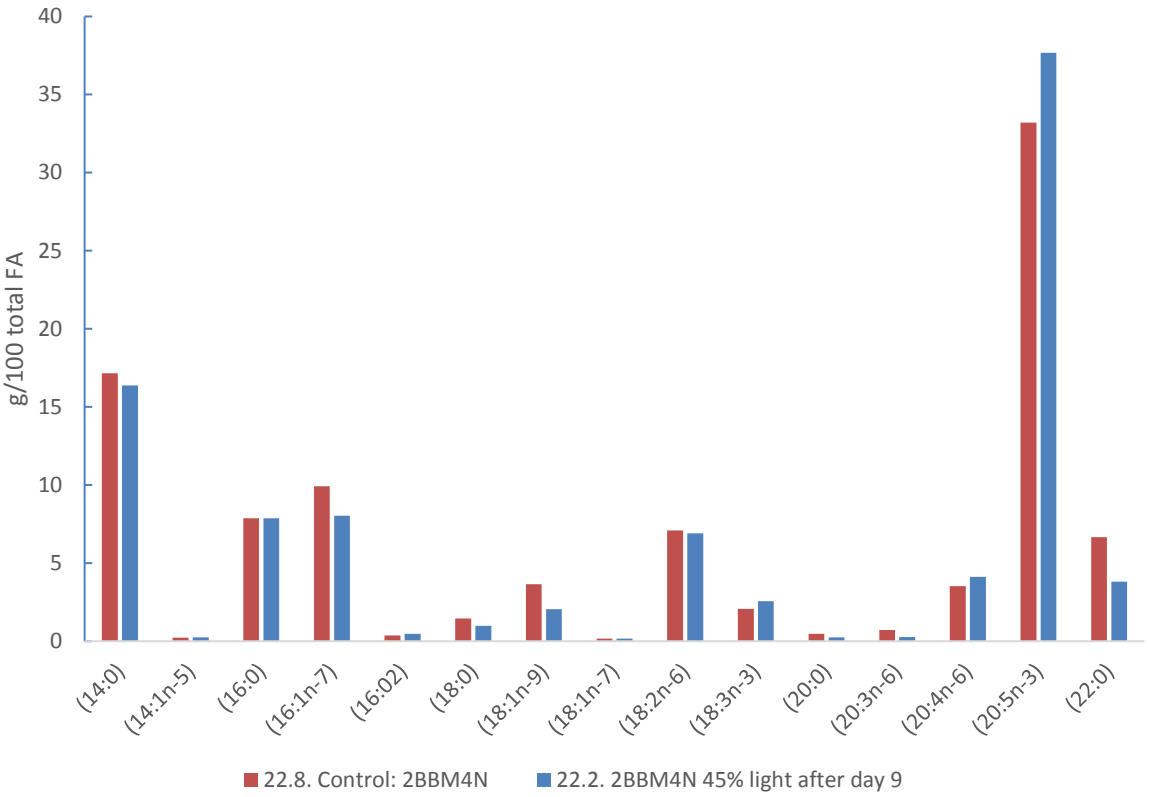

Figure 75. FA profiles from light limited culture

8.4. Conclusions and Recommendations

From this experiment, conclusion about wavelength could not be made because the wavelength of each cellophane was not measured. The hypothesis based on the modelling that there was light limitation was proved. There was no sign of light inhibition, but it seemed that the culture still had light limitation even after the intensities was increased to high light intensity. With high light intensity, the high-density culture needed more light. The light limitation also might be caused by having too many flasks used in the incubator.

A culture with 0.5 g/L sodium nitrate started to starve of nitrogen in day 14. To prevent starvation, it was better to use 1 g/L sodium nitrate in the culture media.

Chapter IX

CONFIRMATION RUNS, OVERALL CONCLUSIONS, AND FUTURE WORK

9.1. Confirmation Runs

9.1.1 Methods

Based on all the experiments that have been done, a confirmation run was performed to compare the final prototype culture medium with the original medium used as basis medium in previous experiments. The prototype culture medium omitted cobalt and boron, and only used one source of phosphate which was monopotassium phosphate. OHM, which was one of the best culture media in Experiment 14, was also tested again in this experiment with four times nitrogen concentration. This meant to ensure that the modified culture medium worked better than OHM. The experiment runs were:
22.1. OHM4N
22.8. 2BBM4N (control)
22.9. 2BBM4N Iron source: $Fe(II)SO_4$ + Na_2EDTA
22.10. 2BBM4N without cobalt
22.11. 2BBM4N without cobalt and boron
23.1. Prototype medium
23.2. 2BBM4N

The flasks used were 250 mL baffled flasks, contained 100 mL culture media. The initial cell density was 2×10^5 cells/mL. The cultivation process took place in the MaxQ™ 6000 incubator. The temperature was maintained at 25 °C. The culture was exposed to high light intensity and shaken at 150 RPM. 3% CO_2 enriched air was supplied into the culture with a total mass flow rate for all flasks of 500 mL/min and a 0.2 µm filter was used to sterilize the gas.

The biomass was harvested by centrifugation at 2907 RCF for 15 minutes. The liquid was disposed then the samples were dried using freeze dryer. The dry samples were sent to Callaghan Innovation for fatty acid analysis.

9.1.2 Results and Discussions

The improved culture medium was compared with OHM (with four times nitrogen concentration) that gave the best growth in the Experiment 14. The production improvement by the modified medium was significantly higher than OHM, up to 2 g/L biomass (Figure 77). In Figure 76, culture medium without cobalt but still with boron produced higher biomass than the control, but the culture medium without cobalt and boron showed the best growth. This confirmed the previous experiment's consideration to remove them from the culture medium.

The fatty acid production was also higher than the control. The total of fatty acid contents was 15% of the biomass with 32.41% EPA in total FA (Table 15).

The 2BBM4N Fe(II)SO_4 Na_2EDTA culture was tested once more to confirm the result of Experiment 17 since it had the best growth in that experiment. There was no significant difference with the control. The FA was lower than 2BBM4N without cobalt and boron but the EPA was slightly higher (34% of the total FA). Overall, there was no significant difference in fatty acid profile between the tested culture medium (OHM biomass fatty acid profile was not analysed).

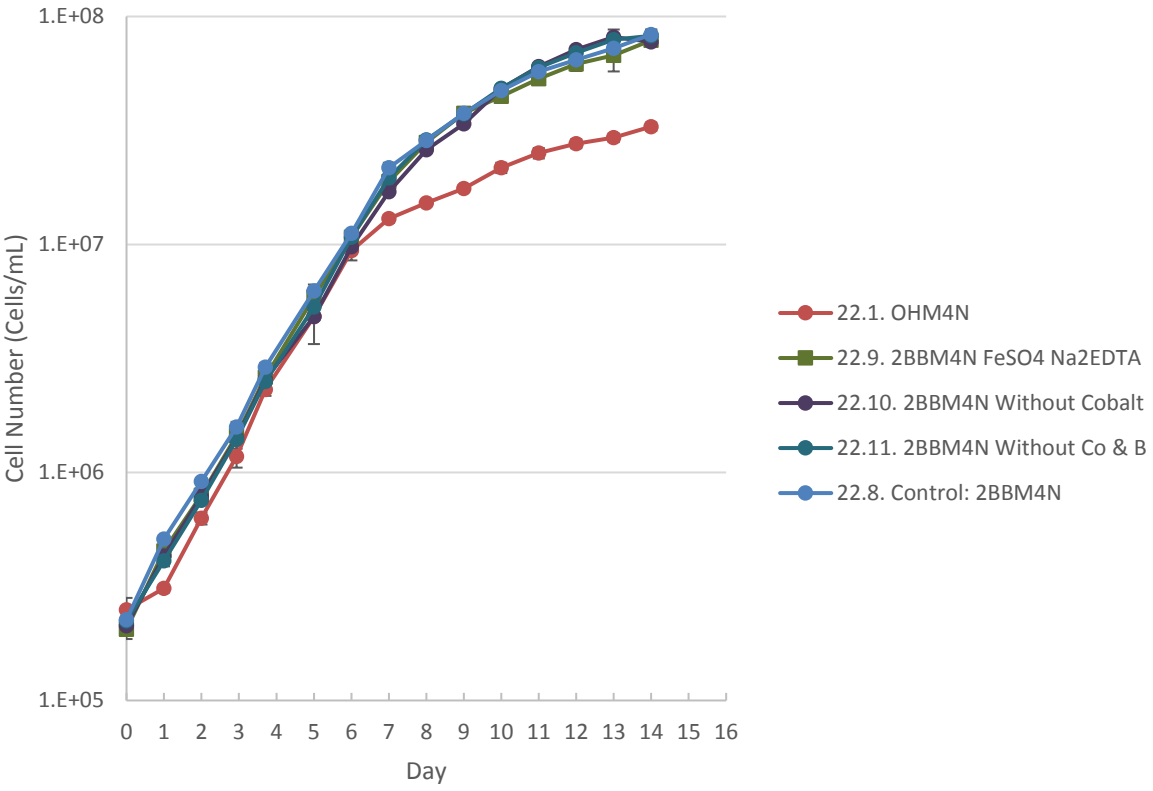

Figure 76. Growth curve of T. sp. LCR-Awa9/2 in 2BBM4N and OHM medium.

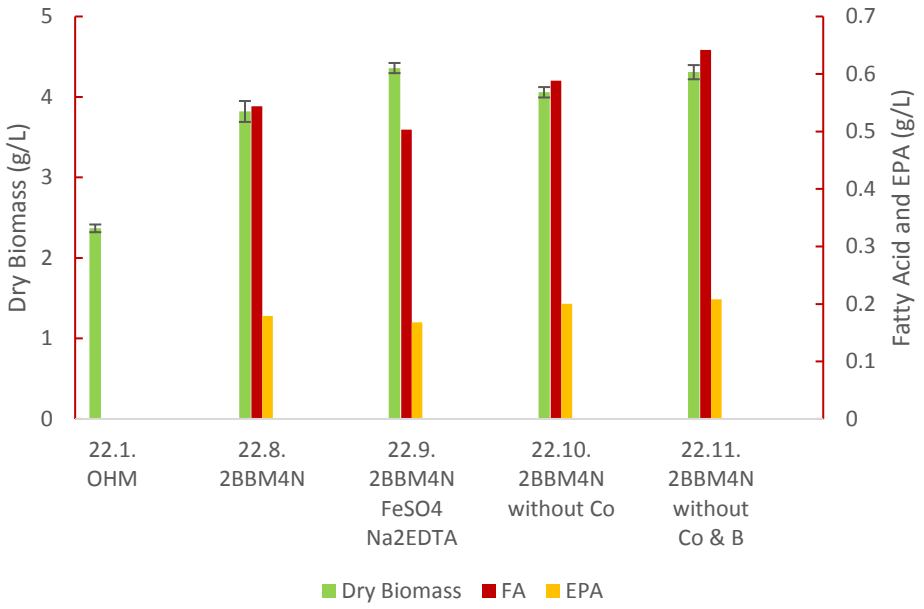

Figure 77. Dry weight of biomass and fatty acid mass from cultivation of *T.* sp. LCR-Awa9/2 from confirmation runs.

Table 16. Fatty acid profile in *T.* sp. LCR-Awa9/2 (Exp. 22).

Fatty Acid g/100g Total FA	22.8. 2BBM4N	22.8. 2BBM4N	2BBM4N $FeSO_4$ Na_2EDTA	2BBM4N without Cobalt and Boric
Myristic acid (14:0)	17.01	17.30	16.55	17.35
Myristoleic acid (14:1n-5)	0.22	0.23	0.16	0.24
Palmitic acid (16:0)	7.85	7.91	7.78	7.64
Palmitoleic acid (16:1n-7)	10.26	9.61	10.55	10.30
16:2	0.40	0.34	0.67	0.33
Stearic acid (18:0)	1.51	1.40	1.55	1.54
Elaidic acid (18:1n-9)	3.71	3.59	2.64	4.56
Vaccenic acid (18:1n-7)	0.16	0.15	0.17	0.21
Linoleic acid (18:2n-6)	7.14	7.03	5.78	6.78
α-Linolenic acid (18:3n-3)	1.98	2.16	2.24	1.87
Arachidic acid (20:0)	0.49	0.47	0.60	0.57
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.70	0.73	0.70	0.72
Arachidonic acid (20:4n-6)	3.40	3.65	2.94	3.13
Eicosapentaenoic acid (**20:5n-3**)	32.97	33.42	34.06	32.41
Behenic acid (22:0)	6.96	6.37	6.79	7.24
Total	**94.77**	**94.36**	**93.18**	**94.88**
FA g/100g DM	**13.9**	**13.5**	**13.5**	**14.9**
FA (g/L)	**0.544**	**0.503**	**0.589**	**0.642**
%FA	**14%**	**14%**	**14%**	**15%**
Dry Mass (g/L)	**3.912**	**3.728**	**4.359**	**4.308**

Table 17. The fatty acid profiles fraction of total fatty acid (Exp. 22).

Fatty Acid, g/100g DM	22.8. 2BBM4N	22.8. 2BBM4N	2BBM4N FeSO4 Na2EDTA	2BBM4N without Cobalt and Boric
Myristic acid (14:0)	2.36	2.33	2.23	2.58
Myristoleic acid (14:1n-5)	0.03	0.03	0.02	0.04
Palmitic acid (16:0)	1.09	1.07	1.05	1.14
Palmitoleic acid (16:1n-7)	1.43	1.30	1.42	1.53
16:2	0.06	0.05	0.09	0.05
Stearic acid (18:0)	0.21	0.19	0.21	0.23
Elaidic acid (18:1n-9)	0.52	0.48	0.36	0.68
Vaccenic acid (18:1n-7)	0.02	0.02	0.02	0.03
Linoleic acid (18:2n-6)	0.99	0.95	0.78	1.01
α-Linolenic acid (18:3n-3)	0.28	0.29	0.30	0.28
Arachidic acid (20:0)	0.07	0.06	0.08	0.09
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.10	0.10	0.09	0.11
Arachidonic acid (20:4n-6)	0.47	0.49	0.40	0.47
Eicosapentaenoic acid (**20:5n-3**)	4.58	4.51	4.60	4.83
Behenic acid (22:0)	0.97	0.86	0.92	1.08
EPA g/L	**0.179**	**0.168**	**0.200**	**0.208**
% EPA of total FA	**5%**	**5%**	**5%**	**5%**

This study identified the key micro nutritional requirements for *T.* sp. LCR-Awa9/2 biomass production. The final prototype of modified culture medium used for further study was Bold Basal medium with four times N concentration, without $Co(NO_3)_2$ and H_3BO_3. The original recipe used $FeSO_4$ and Fe-EDTA, but the prototype used Fe-Na-EDTA instead. The prototype used only one source of phosphate, which was monopotassium phosphate.

Table 18. Bold Basal Modified Awarua medium composition for *T.* sp. LCR-Awa9/2.

Component	Chemical Formula	Mass (g/L)	Concentration (M)
Calcium chloride dihydrate	$CaCl_2.2H_2O$	0.025	1.70E-04
Copper (II) sulphate pentahydrate	$CuSO_4.5H_2O$	0.00157	6.29E-06
Ethylenediaminetetraacetic acid ferric sodium	$C_{10}H_{14}N_2NaFeO_9$	0.063	1.64E-04
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	0.075	6.23E-04
Manganese(II) chloride tetrahydrate	$MnCl_2.4H_2O$	0.00144	9.41E-06
Molybdenum (VI) oxide	MoO_3	0.00071	4.93E-06
Monopotassium phosphate	KH_2PO_4	0.22	1.62E-03
Sodium chloride	NaCl	0.025	4.28E-04
Sodium nitrate	$NaNO_3$	1.00	1.18E-02
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	0.00882	3.07E-05

The modified medium gave higher growth than the 2BBM4N medium (Figure 78). The final cell number was up to 8×10^7 cells/mL.

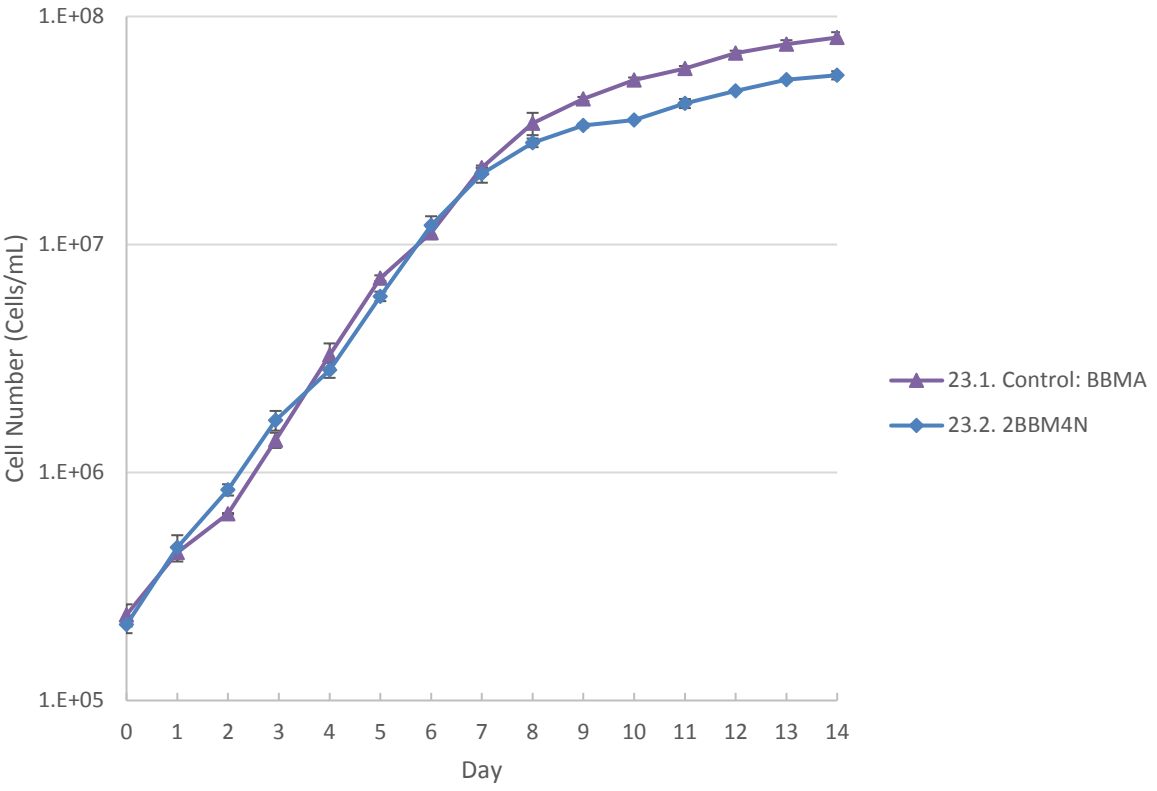

Figure 78. Growth curve of T. sp. LCR-Awa9/2 in BBMA medium.

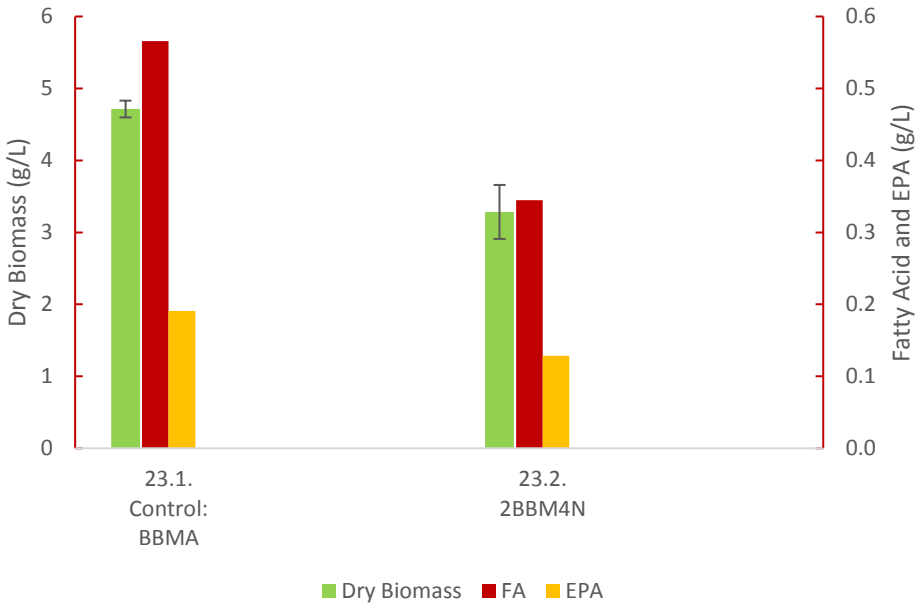

Figure 79. Dry weight of biomass and fatty acid mass from cultivation of T. sp. LCR-Awa9/2 in BBMA.

The fatty acid profile from BBMA and 2BBM4N in Experiment 23 is given in Table 18 and 19. The fatty acid profiles were consistent from the previous experiment.

Table 19. Fatty acid profile in *T.* sp. LCR-Awa9/2 (Exp. 23).

Fatty Acid g/100g Total FA	23.1. Control: BBMA	23.2. 2BBM4N
Myristic acid (14:0)	16.50	16.26
Myristoleic acid (14:1n-5)	0.63	0.92
Palmitic acid (16:0)	7.62	7.91
Palmitoleic acid (16:1n-7)	10.10	8.70
16:02	0.48	0.75
Stearic acid (18:0)	1.23	0.76
Elaidic acid (18:1n-9)	3.64	1.70
Vaccenic acid (18:1n-7)	0.18	0.15
Linoleic acid (18:2n-6)	6.69	6.15
α-Linolenic acid (18:3n-3)	2.17	2.92
Arachidic acid (20:0)	0.54	0.39
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.55	0.51
Arachidonic acid (20:4n-6)	3.44	4.52
Eicosapentaenoic acid (**20:5n-3**)	33.75	37.33
Behenic acid (22:0)	7.22	4.46
Total fatty acids, %	**12.00**	**10.50**

Table 20. The fatty acid composition in 100 g dry mass (Exp. 23).

FA g/100 g DM	23.1. Control: BBMA	23.2. 2BBM4N
Myristic acid (14:0)	1.98	1.71
Myristoleic acid (14:1n-5)	0.08	0.10
Palmitic acid (16:0)	0.91	0.83
Palmitoleic acid (16:1n-7)	1.21	0.91
16:02	0.06	0.08
Stearic acid (18:0)	0.15	0.08
Elaidic acid (18:1n-9)	0.44	0.18
Vaccenic acid (18:1n-7)	0.02	0.02
Linoleic acid (18:2n-6)	0.80	0.65
α-Linolenic acid (18:3n-3)	0.26	0.31
Arachidic acid (20:0)	0.07	0.04
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.07	0.05
Arachidonic acid (20:4n-6)	0.41	0.47
Eicosapentaenoic acid (**20:5n-3**)	4.05	3.92
Behenic acid (22:0)	0.87	0.47
FA g/L	**0.57**	**0.34**
EPA g/L	**0.19**	**0.13**
g EPA/g dry biomass	**0.041**	**0.04**
Dry Mass (g/L)	**4.71**	**3.28**

Experiment 23 was a repetition of Experiment 22. There were four conditions tested: 2BBM4N, ½N, $^{1}/_{40}$P, and Bold Basal Modified LCR-Awa9/2 (BBMA) medium. As shown in Figure 80, the results seemed consistent in both experiment with minor differences. While in Figure 81, the 2BBM4N in Experiment 23 was lower than the previous experiment. Overall, the cell production in Experiment 23 was lower than Experiment 22. There were some problems caused this, first was the gas filters needed to be renewed. They have been used for a year and became

wet easily. In later experiments, each filter had to be replaced with the dry one (it was dried in an oven, at 80 °C) every day, but still some flask did not get any gas bubbles for some hours (less than 12 hours because the cultures were monitored every 12 hours). Second, some of the cultures were getting more viscous day by day. The different viscosity (observed), and possibly surface tension between flasks caused uneven gas distribution. Even with the highest total gas flowrate, the gas was not bubbling into all flask. Third, the volume differences in this experiment were significant. 15 mL samples were taken from some flasks resulting in different gas distribution in each flask. Reaching 8×10^7 cells/mL, with 18 to 22 flasks in the incubator at high light intensity and maximum gas flow rate, was the best that the incubator could do. The disturbed gas did not occur in Experiment 22 because there were 6 flasks harvested on day 8, so there were only 12 flasks in the incubator afterwards.

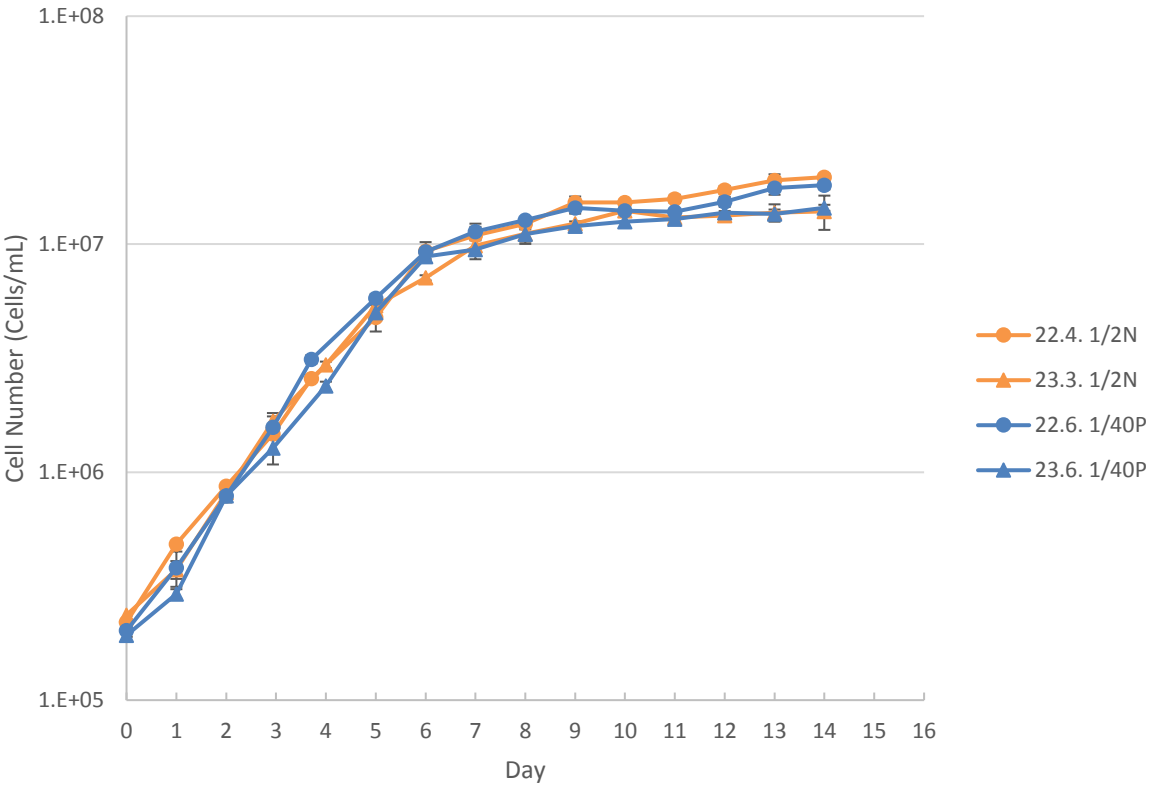

Figure 80. The growth curve of *T.* sp. LCR-Awa9/2 in BBMA with N and P starvation in Experiment 22 and 23.

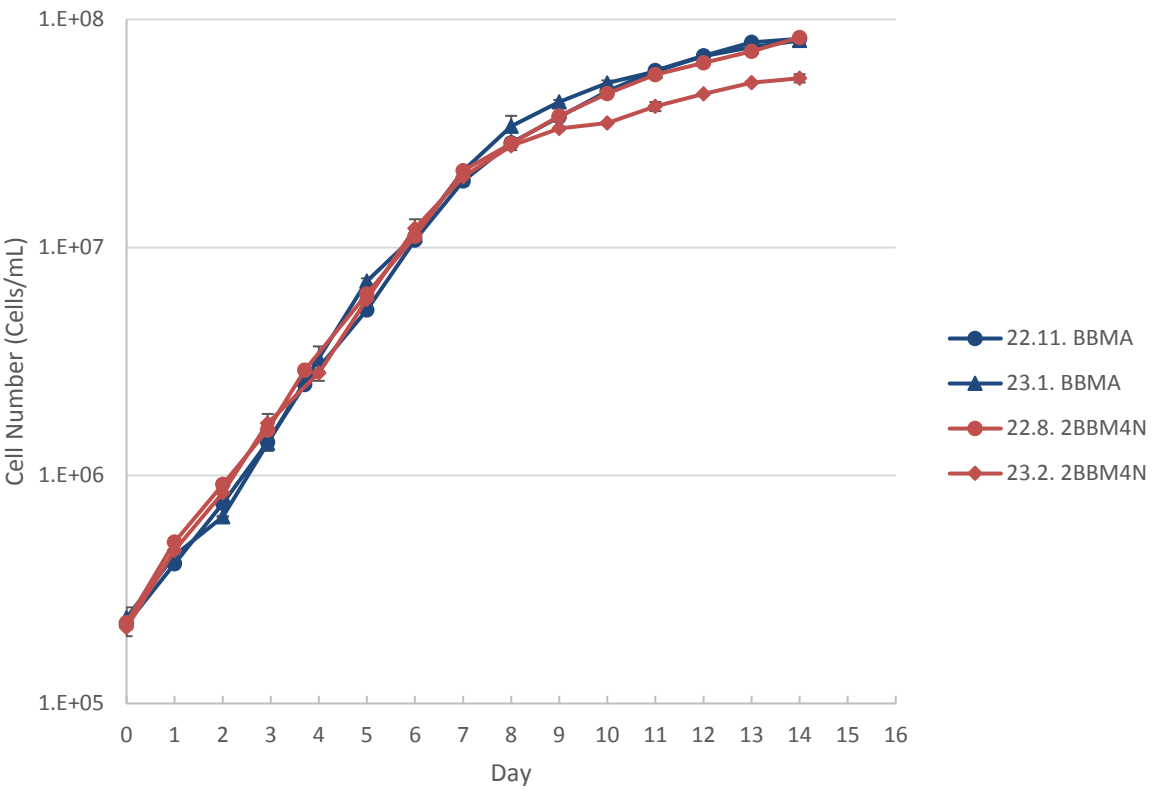

Figure 81. The growth curve of *T.* sp. LCR-Awa9/2 in 2BBM4N and BBMA medium in Experiment 22 and 23.

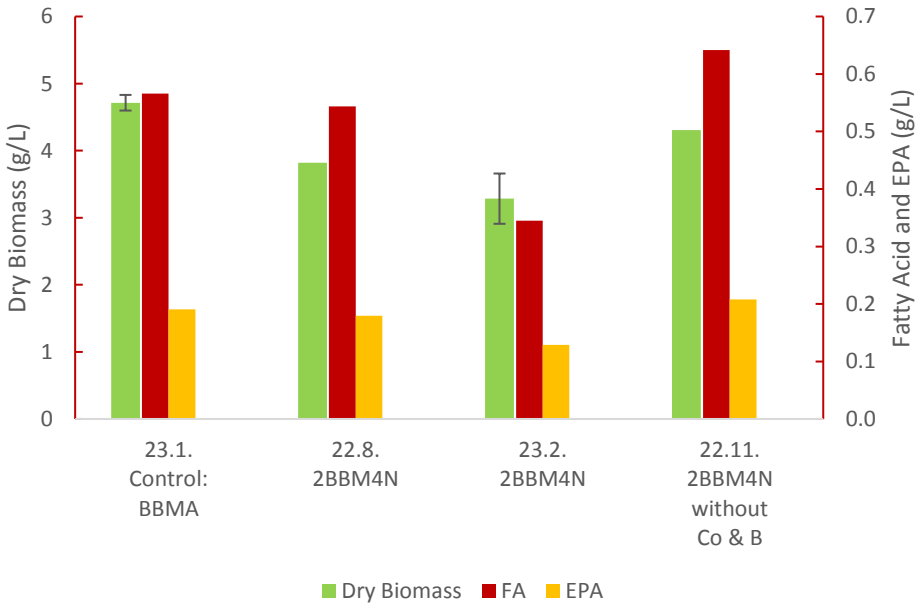

Figure 82. The biomass dry weight of *T.* sp. LCR-Awa9/2 in 2BBM4N and BBMA medium in Experiment 22 and 23.

9.2. Overall Conclusions

There were 25 experiments in this project. Experiments 1-13 were preliminary experiments where the methods for the project were improved. The base culture medium was Bold Basal medium and determined from Experiment 14 in Chapter IV.

The microalgal could tolerate sodium chloride with a concentration up to 2 g/L. It was found that total ionic concentration did not affect the microalgal growth. Microalgal culture in media with different osmotic pressure had similar growth. Culture in twice and four times concentration of Bold Basal medium also had slight differences in the growth curve. It was clear that the concentration of individual chemicals plays a role in the microalgal growth. Boron, zinc, and sulfate were suspected to be toxic in six times concentration of BBM.

Next experiment was done to investigate the limiting nutrient that inhibited the growth in ¼BBM, ½BBM, and BBM. Nitrogen was the limiting nutrient. Adding more boric acid to the culture gave a toxic environment to the microalgal culture.

In Chapter V, sodium nitrate was found to be more desirable by the microalga in term of cell number, dry biomass, fatty acid, and EPA production. Urea seemed to be effective for the microalga but with lower productivity than sodium nitrate. Adding nickel in urea culture medium gave higher growth than urea culture medium without nickel.

The role of trace elements was discussed in Chapter VII. Cobalt and boron were demonstrated to be slightly toxic to the microalgal culture. Supplementing the culture with selenium and vitamin did not improve the microalgal growth. Culture medium with selenium dioxide had better growth than selenous acid. Using different sources of iron and chelating agent did not show considerable improvement in growth.

Nitrogen, phosphate, and complete starvation increased the amount of fatty acid in dry biomass. However, they stimulated more elaidic acid than EPA. Sudden light reduction in day 8 produced 37.7% EPA in total FA, 5% higher than the stress-free culture. Through the dynamic modelling of the microalgal growth, a light limitation was found in medium light intensity and started on the fifth day of the cultivation. The light intensity then increased to high intensity to improve the growth. Through the modelling, the nitrogen and phosphate consumption ratio were also calculated, 0.1113 gNO_3^-/g biomass and 0.00043 gPO_4^{3-}/g biomass.

The final prototype medium from this study was named Bold Basal Modified Awarua (BBMA) medium. The highest EPA production was obtained by the BBMA which was 0.208 g EPA/L culture or 0.048 g EPA/g biomass. The biomass production was 4.3 g/L and lipid 0.64 g/L culture or 0.15 g FA/g biomass.

The sodium nitrate concentration in BBMA was increased to four times higher than the original recipe. The BBMA only used one source of phosphate which is monopotassium phosphate. Cobalt chloride and boric acid were removed from the culture medium recipe.

In experiment 14, the culture in BBM produced 2.35±0.06 g/L biomass. After modification, microalgal culture in BBMA could produce 4.7±0.1 g/L biomass. Using only two flasks in the incubator with less light interference, the microalgal culture in BBMA obtained 9×10^7 cells/mL with 5.6±0.15 g/L dry biomass (Experiment 24, Appendix D).

9.3. Future Work

It should be possible to reach 1×10^8 cells/mL culture by making some improvements: increase the light intensity (the more populated the cells, the light limiting problem occurred) and find the optimum light intensity required to avoid photoinhibition, increase the gas mass flow because the more viscous the culture the gas became more difficult to be distributed evenly in all flasks in the incubator. Shake flasks are probably a limitation to effective light penetration. High growth rates might require a different physical configuration with short light path lengths.

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APPENDIX A

CONSUMABLES AND EQUIPMENT

Below are consumables needed in the experiment. All consumables are provided by the University of Canterbury.

- Ammonium ferric citrate ($C_6H_{11}FeNO_7$)
- Ammonium chloride (NH_4Cl)
- Ammonium metavanadate (NH_4VO_3)
- Boric acid (H_3BO_3)
- Calcium chloride dehydrate ($CaCl_2.2H_2O$)
- Citric acid ($C_6H_8O_7$)
- Cobalt (II) chloride ($CoCl_2$)
- Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)
- Cobalt (II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$)
- Cobalt (II) sulphate heptahydrate ($CoSO_4.7H_2O$)
- Copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$)
- EDTA ($C_{10}H_{16}N_2O_8$)
- Ethylenediaminetetraacetic acid ferric sodium (EDTA, Fe Na)
- Ethylenediaminetetraacetic acid, disodium salt ($Na_2C_{10}H_{14}N_2O_8.2H_2O$)
- Hexaammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)
- Hydrochloric acid (HCl)
- Iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$)
- Iron (III) citrate tribasic monohydrate ($C_6H_5FeO_7.H_2O$)
- Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)
- Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)
- Manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$)
- Manganese (II) sulphate tetrahydrate ($MnSO_4.4H_2O$)
- Methanol
- Molybdenum (VI) oxide (MoO_3)
- Monopotassium phosphate (KH_2PO_4)
- Nickel (II) sulphate hexahydrate ($NiSO_4.6H_2O$)
- Potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$)
- Potassium nitrate (KNO_3)
- Selenium dioxide (SeO_2)
- Selenous acid (H_2SeO_3)
- Sodium carbonate (Na_2CO_3)
- Sodium chloride (NaCl)
- Sodium hydrogen carbonate ($NaHCO_3$)

- Sodium hydrogen phosphate (Na_2HPO_4)
- Sodium molybdate dehydrate ($NaMoO_4.2H_2O$)
- Sodium nitrate ($NaNO_3$)
- Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)
- Thiamine HCl
- Biotin
- Vitamin B_{12}
- Ultra-pure deionized water (H_2O)
- Ethanol
- Eppendorf tubes
- Pipette tips (1000 and 200 µL)
- Falcon tubes
- Filter paper 0.7 µm pore diameter
- Autoclave tape
- Cotton
- Aluminium foil
- Gloves
- Syringe filters 0.22 µm pore diameter
- Syringe
- Kimtech tissue

Below is the equipment that have been used in the experiment.

- Assorted rubber stoppers
- Autoclave
- Baffled Erlenmeyer flasks
- Beaker glass
- Biological cabinet
- Centrifuge
- Digital balance
- Erlenmeyer
- Freeze dryer
- Funnel
- Glass bottle
- Graduated cylinder
- Magnetic stirrer
- Micropipette
- Microscope
- Oven
- Pipette bulb
- Scoopula
- Shaker incubator
- Spatula
- Volumetric flask
- Volumetric pipettes
- Wash bottle

APPENDIX B

CULTURE MEDIA RECIPE

Zehnder Medium (Z) (Alexandrov et al., 2014)

- Prepare the micronutrient solution. To 100 mL of water add the following chemicals, fully dissolve between each addition, and then make it up to 250 mL in a volumetric flask.

Component	Quantity (g)
H_3BO_3	1.545
$MnSO_4.4H_2O$	0.5575
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.49
$ZnSO_4.7H_2O$	0.7175
$CuSO_4.5H_2O$	0.62225
$CoCl_2$	0.75

- Into 500 ml of water add the following stock solution, fully dissolve between each addition, and then make it up to 1000 mL in a volumetric flask.

Component	Quantity (g)
$NaNO_3$	0.467
$CaCl_2$	0.04
K_2HPO_4	0.031
$MgSO_4.7H_2O$	0.025
Na_2CO_3	0.021
Fe-EDTA complex	0.01
micronutrient solution **	1 mL

- Store the liquid in a glass bottle.
- Autoclave at 121°C for 15 minutes.
- Store it in a refrigerator.

MLA Medium (MLA) (Bolch & Blackburn, 1996)

– Prepare vitamin stock solutions.
  - Make primary stocks (each made up separately):

Component	Stock Solution (mg/100 mL H2O)
Biotin	10
Vitamin B12	10

  - To 50 ml of water add the following chemicals, fully dissolve between each addition, and then make it up to 100 mL in a volumetric flask.

Component	Quantity
Thiamine HCl	10 mg
Biotin	0.05 mL primary stock
Vitamin B_{12}	0.05 mL primary stock

– Prepare micronutrient stock solution
  - To 800mL of water add each of the following constituents, mixing to dissolve each addition (Add Na_2EDTA first & stir on low heat until fully dissolved).

Component	Quantity (g)
Na_2EDTA	4.36
$FeCl_3.6H_2O$	1.58
$NaHCO_3$	0.6
$MnCl_2.4H_2O$	0.36

  - Add 10 mL of the following primary stocks (each made up separately):

Component	Quantity (g/L H_2O)
$CuSO_4.5H_2O$	1
$ZnSO_4.7H_2O$	2.2
$CoCl_2.6H_2O$	1
$Na_2MoO_4.2H_2O$	0.6

  - Make up the micronutrient stock to 1000 mL with water using volumetric flask.
  - If precipitate forms increase pH up to 7. (If precipitation becomes an issue then replacing the two sulphate stocks with equimolar amounts of the trace metal in the chloride form has proven useful).

- Prepare macronutrient stock solutions (each made up separately):

Component	Stock Solution (g/L H_2O)
$MgSO_4.7H_2O$	49.4
$NaNO_3$	85
K_2HPO_4	6.96
H_3BO3	2.47
H_2SeO_3	1.29

- Prepare $NaHCO_3$ solution (16.9 g/L H_2O). Autoclave to sterilise.
- Prepare $CaCl_2.2H_2O$ solution (29.4 g/L H_2O). Autoclave to sterilise.
- Prepare MLA medium x40 concentrated nutrients (250mL volume)
  - Combine the stock solutions below and then make up to 250 mL in volumetric flask by adding water.

Component	Quantity (mL)
$MgSO_4.7H_2O$	10
$NaNO_3$	20
K_2HPO_4	50
H_3BO_3	10
H_2SeO_3	10
Vitamin stock	10
Micronutrient stock	10

  - Filter sterilise using a 0.22 mm filter into a sterile 250 mL Schott bottle.

- Combine the stock solutions below and then make up to 1000 mL in a sterile volumetric flask by adding sterile water.

Component	Quantity (mL)
Sterile MLA medium x40 concentrated nutrients	25
Sterile $NaHCO_3$	10
Sterile $CaCl_2.2H_2O$	1

- Transfer the liquid into a sterile glass bottle.
- Store it in the refrigerator.

Bold Basal Medium (BB) (Australian National Algae Culture Collection)

- Prepare macronutrient stock solutions (each made up separately):

Component	Stock Solution (g/L H_2O)
$NaNO_3$	25
$CaCl_2.2H_2O$	2.5
$MgSO_4.7H_2O$	7.5
K_2HPO_4	7.5
KH_2HPO_4	17.5
NaCl	2.5

- Prepare additional stock solutions (each made up separately):

Component	Stock Solution (g/L H_2O)
Fe-Na-EDTA	5
H_3BO_3	11.42

- Prepare micronutrient stock solution. Add each constituent separately to ~800 mL of water and fully dissolve between each addition. Then make up to 1000 mL in volumetric flask.

Component	Quantity (g)
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

- Add 10 mL of each macronutrient stock solution, and 1 mL of additional and micronutrient stock solutions, and then make up to 1000 mL in volumetric flask by adding water.
- Transfer the liquid into a sterile glass bottle.
- Autoclave at 121°C (15PSI for 15 mins).
- Store it in the refrigerator.

Blue Green 11 Medium (BG11) (Department Experimental Phycology and Culture Collection of Algae (EPSAG), 2013)

- Prepare $NaNO_3$ solution (15 g/L dH_2O)
- Prepare macronutrient stock solutions, each made up separately.

Component	Stock Solution (g/L H_2O)
K_2HPO_4	4
$MgSO_4.7H_2O$	7.5
$CaCl_2.2H_2O$	3.6
Citric Acid	0.6
Ferric Ammonium Citrate	0.6 (Autoclave to dissolve)
Na_2EDTA	0.00127
Na_2CO_3	2

- Prepare micronutrient stock solution. Add each constituent separately to ~800 mL of water and fully dissolve between each addition. Then make up to 1000 mL in volumetric flask.

Component	Stock Solution (g/L H_2O)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$NaMoO_4.H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

- Combine 100 mL of $NaNO_3$ solution with 10 mL of each macronutrient solutions and 1 mL of the micronutrient solution. Make up to 1000 mL by adding water into a volumetric flask.
- Transfer the liquid into a sterile glass bottle.
- Autoclave at 121°C for 15 mins.
- Store it in the refrigerator.

Zachleder and Setlik Medium (½SS) (Zachleder & Setlík, 1982)

- Prepare <u>micronutrient stock solution</u>. Add each constituent separately to ~800 mL of water and fully dissolve between each addition. Then make up to <u>1000 mL</u> in volumetric flask.

Component	Stock Solution (g/L H_2O)
H_3BO_3	3.086
$MnSO_4.4H_2O$	1.18
$CoCl_2.7H_2O$	0.136
$CuSO_4.5H_2O$	1.244
$ZnSO_4.7H_2O$	1.43
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.84

- Prepare stock solutions A:

Component	Quantity/ L H_2O
$MgSO_4.7H_2O$	98.8 g
Micronutrient stock solution	100 mL

- Prepare stock solutions B:

Component	Stock Solution (g/L H_2O)
KNO_3	202
$CaCl_2.6H_2O$	1.1
Fe-Na-EDTA	2

- Prepare stock solutions C:

Component	Stock Solution (g/L H_2O)
KH_2PO_4	34

- Combine 10 ml of solution A, B, and C, and make it up to 1000 mL by adding water into a volumetric flask.
- Transfer the liquid into a sterile glass bottle.
- Autoclave at 121°C for 15 mins.
- Store it in the refrigerator.

Optimum Haematococcus Medium (Fábregas et al., 2000)

- Prepare vitamin stock solutions. To 50 mL of water, add the following vitamins. Make it up to 100 mL in a volumetric flask.

Component	Stock Solution (mg/100 mL H_2O)
Biotin	2.5
Vitamin B_{12}	1.5
Thiamine HCl	1.75

- Prepare macronutrient stock solutions (each made up separately):

Component	Stock Solution (g/100 mL H_2O)
KNO_3	4.1
Na_2HPO_4	0.3
$MgSO_4.7H_2O$	2.46
$CaCl_2.2H_2O$	1.1

- Prepare micronutrient stock solution. Add each constituent to ~800 mL of dH_2O and fully dissolve between each addition. Then make up to <u>1000 mL</u> by adding water in a volumetric flask.

Component	Stock Solution (mg/L H_2O)
Fe(III)Citrate.H_2O	5.24
$CoCl_2.6H_2O$	0.022
$CuSO_4.5H_2O$	0.024
Cr_2O_3	0.152
$MnCl_2.4H_2O$	1.978
$Na_2MoO_4.2H_2O$	0.24
SeO_2	0.01

- Combine 10 mL of each macronutrient stock solution, 1 mL vitamin, and 0.5 mL of micronutrient stock solution.
- Filter the solution using syringe filter pore size 0.2 µm into a sterile volumetric flask. Then make it up to 1000 mL by adding sterile water.
- Transfer the liquid into a sterile glass bottle.
- Store it in the refrigerator.

Modified Bourelly Medium (Krienitz & Wirth, 2006)

- Prepare vitamin stock solutions. To 50 mL of water, add the following vitamins. Make it up to 100 mL in a volumetric flask.

Component	Stock Solution (mg/100 mL H_2O)
Biotin	3.3
Vitamin B_{12}	0.5
Thiamine HCl	0.5

- Prepare macronutrient stock solutions (each made up separately):

Component	Stock Solution (g/100 mL H_2O)	Applied Solution (mL)
KNO_3	10	2
K_2HPO_4	1	4
$MgSO_4.7H_2O$	1	3
$Ca(NO_3)_2$	1	3
$NaHCO_3$	1.68	10
Fe-EDTA	0.35	1

- Prepare micronutrient stock solution. Add each constituent to ~800 mL of dH_2O and fully dissolve between each addition. Then make up to 1000 mL by adding water in a volumetric flask.

Component	Stock Solution (mg/L H_2O)
$MnCl_2.4H_2O$	0.5
$CoSO_4.7H_2O$	0.014
$CuSO_4.5H_2O$	0.003
$ZnSO_4.7H_2O$	0.036
H_3BO_3	0.00155
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.009
$NiSO_4.6H_2O$	0.013
NH_4VO_3	0.0075

- Combine each macronutrient stock solution and 10 mL of each micronutrient stock solution and vitamin.
- Filter the solution using syringe filter pore size 0.2 µm into a sterile volumetric flask. Then make it up to 1000 mL by adding sterile water.
- Transfer the liquid into a sterile glass bottle.
- Store it in the refrigerator.

New Medium by Svetoslav et al. (Alexandrov et al., 2014)

- Prepare the micronutrient solution. To 50 ml of water add the following chemicals, fully dissolve between each addition, and then make it up to 100 mL in a volumetric flask.

Component	Stock Solution (mg)
H_3BO_3	200 mg
$MnSO_4.4H_2O$	200 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.4 mg
$ZnSO_4.7H_2O$	200 mg
$CuSO_4.5H_2O$	4 mg
$CoSO_4.5H_2O$	0.04 mg

- Add each constituent to ~800 mL of water and fully dissolve between each addition. Then make up to 1000 mL in a volumetric flask.

Component	Stock Solution (mg)
$NaNO_3$	2000
$Ca(NO_3)_2.4H_2O$	40
K_2HPO_4	250
$MgSO_4.7H_2O$	266
$NaHCO_3$	100
NaCl	50
$Fe_2(SO_4)_3.7H_2O$	20
Micronutrient solution (ml)	1

- Transfer the liquid into a sterile glass bottle.
- Autoclave at 121°C for 15 mins.
- Store it in the refrigerator.

Bold Basal Modified Awarua (BBMA) Medium

– Prepare macronutrient stock solutions (each made up separately):

Component	Stock Solution (g/L H_2O)
$NaNO_3$	100
$CaCl_2.2H_2O$	5
$MgSO_4.7H_2O$	15
KH_2HPO_4	44
NaCl	5
Fe-Na-EDTA	12

– Prepare micronutrient stock solution. Add each constituent separately to ~800 mL of water and fully dissolve between each addition. Then make up to 1000 mL in volumetric flask.

Component	Quantity (g)
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57

– Add 10 mL of each macronutrient stock solution, and 2 mL of micronutrient stock solution, and then make up to 1000 mL in volumetric flask by adding water.
– Transfer the liquid into a sterile glass bottle.
– Autoclave at 121°C (15PSI for 15 mins).
– Store it in the refrigerator.

APPENDIX C

PRELIMINARY EXPERIMENTS

The preliminary experiments were conducted from 14 December 2016 until June 2017 to develop method and experiment expertise. These results are included to help other researchers who are new to algae culturing.

Experiment 1 (14 December 2016)

The first test started on 14 December 2016. The aim was practicing microalgae cultivation. The microalgae strain, *Trachydiscus* sp. LCR-Awa9/2 was from Landcare Research. The procedure of cell counting is provided in Chapter III. Calculating the strain cell number was the first thing to do. A predetermined volume of strain was transferred into two sterile flasks containing 50 mL MLA medium. The cultivation was done in duplicates, in an incubator shaker with a light intensity of 60 µmol photons/m²s, at 80 RPM, and at room temperature. The initial cell density for both flasks was 2×10^5 cells/mL.

The cell growth was monitored every day for ten days. 1 mL samples were taken from each flask at 1 PM every day. The graph below shows cell growth during the first experiment.

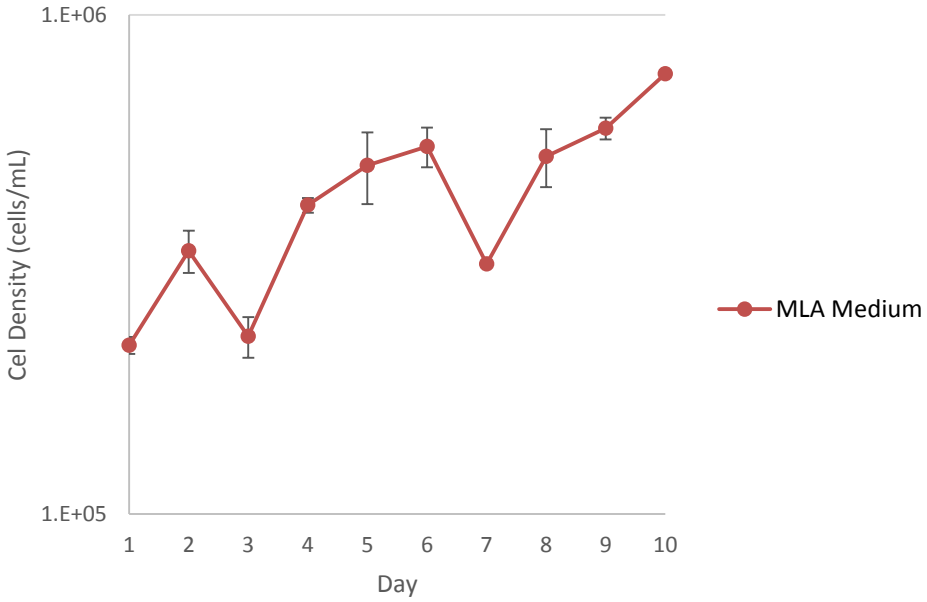

Figure 83. *T.* sp. LCR-Awa9/2 growth curve

In day 10, the cell density was 7.63×10^5 cells/mL. Overall, the growth rate was 0.14/day. The growth curve showed two decreases on day 3 and day 7. There are some concerns in this experiment. First was the temperature. The incubator used in this experiment did not provide a closed system and the temperature was not adequately controlled. The temperature during the

cultivation varied between 24 and 27 ˚C. Evaporation as a result of poor temperature control caused reduction in the volume of the culture media in the flask.

The second problem was cell distribution. There are two main reasons for this. The speed used for this experiment was 80 RPM. At this speed, cells became agglomerated because they were shaken at low RPM. The type of flask was a further cause of error. Ordinary flasks were used for this experiment. This resulted in cells adhering to the Erlenmeyer's wall. The improperly distributed cells caused difficulty in cell counting. The cells were granulated, making them difficult to count.

1 mL samples were collected from each flask using volumetric pipettes. Using volumetric pipettes would not be suitable for working with a large number of variables since it requires one pipette for each sample to prevent contamination. A 1 mL sample was also too large for sampling because it would reduce the volume which could affect the growth.

The time span between sampling and cell counting also determined the error percentage of the cell counting result. The longer it was, the harder to count the cells because they settled easily at the bottom of the tubes. The sampling was done in Von Haast 236 while the cell count was in SP Lab. It took approximately five minutes to travel to Special Purpose Laboratory. The sampling for multiple flasks was performed at a time due to this distance. When the microalgae was cultivated in flask A and flask B, sample was taken from flask A and B in the laminar flow which was in Von Haast 236. Then samples A and B were transported to the SP Lab to be counted. It is suggested to do one sampling at a time, which means a volume of sample is taken from flask A, then count the cell immediately after the sampling, then continue to sample B. This procedure was not conducted in the first experiment because of the separate labs.

In the first experiment, the samples were not homogenized using micropipettes. This resulted in cell counting difficulty because the cells colonized at the bottom of the tubes. Another cause for cell counting difficulties was the type of tissue used for cleaning the hemacytometer. Ordinary tissue was fibrous, and the fiber adhered readily to the hemacytometer, made it appeared in microscope view. Instead, Kimtech tissue was used.

In conclusion, moderate cell growth in this experiment was mainly caused by improper distribution, uncontrolled temperature, and errors on cell counting which were showed by day three and seven drops.

Experiment 2 (12 January 2017)

The second experiment used 50 mL of MLA and BG11 in 250 mL baffled flasks. The cultivation was run at room temperature and 60 µmol photons/m²s light intensity. At the outset, the cell density was 2×10^5 cells/mL. The shaker speed was varied between 65 to 150 RPM since the best speed needed to be investigated to prevent agglomerate and adherence to the wall cells.

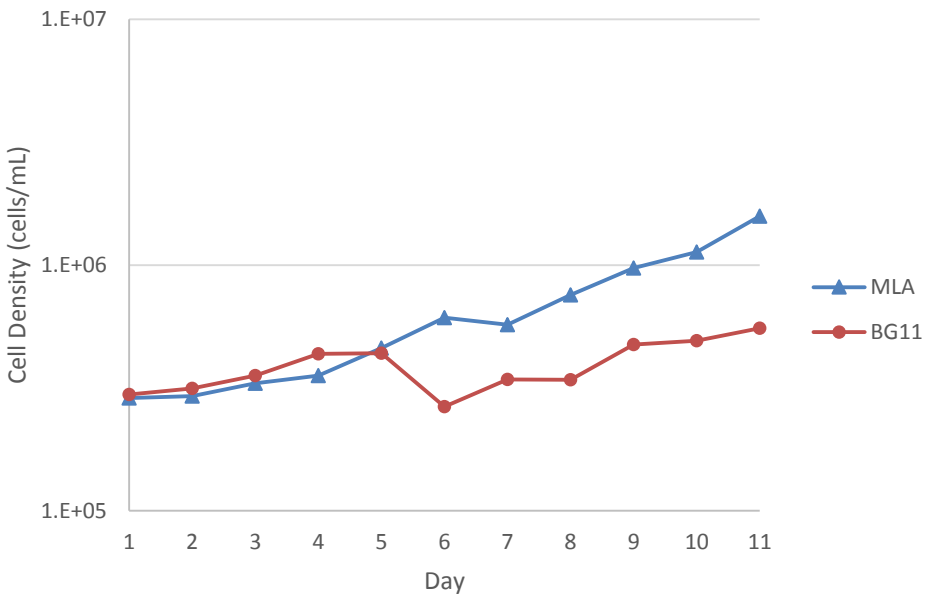

Figure 84. *T.* sp. LCR-Awa9/2 growth curve on MLA and BG11

The result of the second experiment was better than in the previous experiment. The cell density in MLA medium at day eleven was 1.58×10^6 cells/mL. The growth rate of MLA was 0.19/day. Every second day, the shaker speed was changed in a range of 65 to 150 RPM. When the speed was lower than 100 RPM, the cells became colonized. At 100 RPM, the cells distribution was improved but adherence of cells to the flask's wall was still a problem. The optimal speed for the cell distribution was 150 RPM, because although some cells still adhered to the flask's wall, they were not as many as at 100 RPM or lower.

Cell counting was still difficult to do because of the clumps cell as a consequence of cell distribution obstruction because the incubator was set at low RPM.

BG11 did not perform well in this experiment. The overall growth rate was 0.07/day, but after day five, the cell number dropped and then started to rise again at day seven. This might be caused by cell counting errors.

Aside from cell counting errors, there were other issues (shared by the previous experiment) including uncontrolled temperature resulting in evaporation, sample homogenization, and the span time between sampling and cell counting.

0.5 mL samples were taken using a 1000 µL micropipette. The type of tissue used to clean the hemacytometer was replaced by a Kimtech tissue. Sample homogenization was conducted before the small volume of sample was injected into the hemacytometer, as explained in Appendix C. Sampling was done for both flasks at one time and the time span between sampling and cell counting was more than 15 minutes due to the distance of laminar flow and the microscope.

Experiment 3 (24 January 2017)

The third experiment used MLA and BG11, each 100 mL in duplicates with an initial cell density 2×10^5 cells/mL. BG11* in the experiment was environmental microalgae in a flask with cotton plug only (without aluminium foil seal). This was done in order to see if there was any difference between the Erlenmeyer with and without the aluminium foil seal. The experiment was run at room temperature and 150 RPM, with a light intensity of 60 µmol photons/m²s. The sampling used 1000 µL micropipette and a 0.5 mL sample was taken from each flask every day.

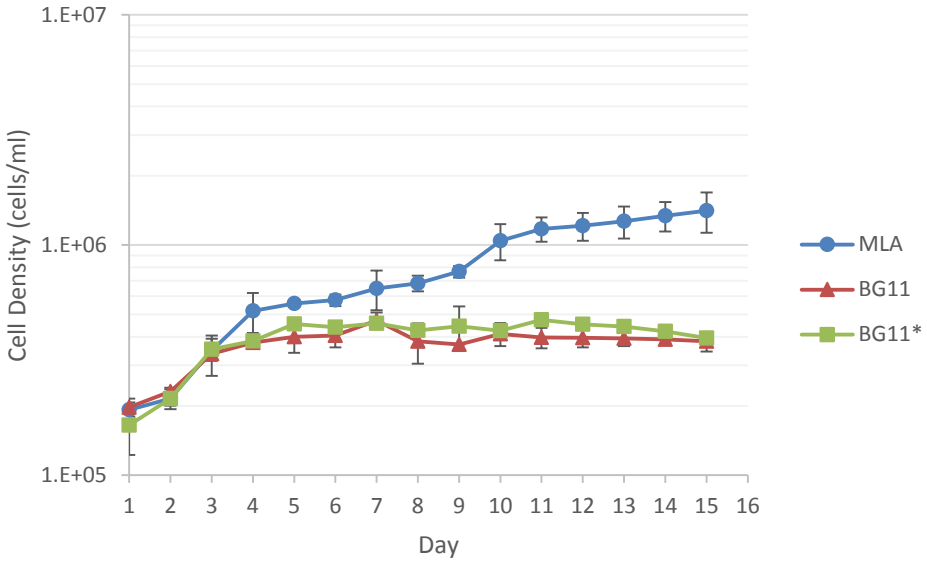

Figure 85. *T.* sp. LCR-Awa9/2 growth curve on MLA and BG11

The results of the third experiment were similar to those of the second experiment. The highest cell density was achieved by MLA on day fifteen with a growth rate of 1.41×10^6 cells/mL with growth rate 0.14/day. In contrast, BG11 entered its stationary phase at day four. This was shown not only by the cell density, but also by the colour change. Environmental algae with MLA medium turned greener every day, while BG11 stopped changing colour after day four. BG11 and BG11* growth rates were 0.05/day and 0.06/day respectively.

As in the previous experiments, there were three problems with the cultivation process and sampling procedure. Firstly, evaporation took place due to a high temperature, reaching up to 30 ˚C. Secondly, the cell numbers on the first day were lower than the expected initial cell density of 2×10^5 cells/mL. This was because the strain used to start the microalgae environment was twenty five days old, therefore, some dying cells might have been calculated during the cell counting. Finally, the time span between sampling and cell counting was the same as previous experiments.

A 0.5 mL sample from each flask was taken daily using a 1000 µL micropipette. Culture homogenization was done twice as described in Chapter III. Before taking the sample from the flask, gentle orbital movement was applied to the flask for 60 seconds. Second homogenization was done before injecting the sample into the hemacytometer. Using a 1000 µL micropipette,

the sample in the Eppendorf tube was pulled into the micropipette and pushed back into the tube without producing any bubbles. This process was repeated sixty times. Then a small volume of sample was injected into the hemacytometer using a 200 µL micropipette. Cell clumping was no longer a concern in this experiment. The cell view under the microscope was clear, without any artifact, dust, or tissue fibre.

There was no significant difference between BG11 sealed with aluminium foil or with only cotton, and no contamination was found in the BG11 sealed with cotton. An alteration was found in the pH level before and after the cell growth. The initial pH of MLA was 7, and after fifteen days the pH changed to 9.623. In contrast, BG11 with an initial pH of 7.3 showed a slight change to 7.4. This indicated that a high rate of cell growth resulted in pH alteration.

Experiment 4 (10 February 2017)

Experiment 4 was performed using ½ SS medium with a total ion concentration of 0.00657 M, and BG11 medium with a total ion concentration of 0.00657 M. The aim of this experiment was to test on ½ SS medium and to examine whether total ionic concentration (TIC) affected the cell growth. Both ½ SSM and BG11 were diluted to MLA total ionic concentration to create a comparison of three different culture media with the same total ionic concentration.

Four flasks were prepared for the experiment, using two culture media in duplicates. At commencement, the initial cell density was 2×10^5 cells/mL. The shaking speed was 150 RPM, at a light intensity of 60 µmol photons/m²s, and at room temperature. The sampling was done with four flasks at a time. A 0.5 mL sample was taken from each flask every day, using a 1000 µL micropipette. The time span between sampling and cell counting was the same as in previous experiments.

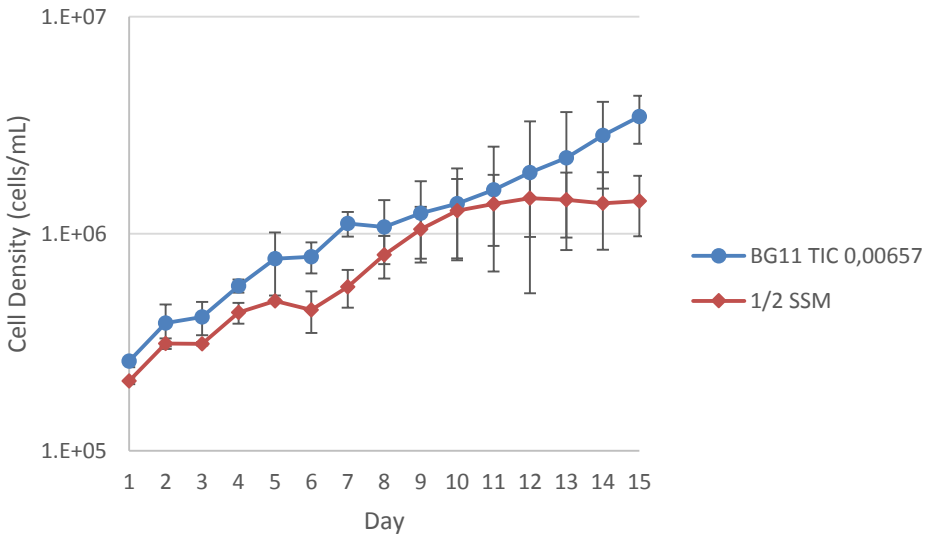

Figure 86. *T.* sp. LCR-Awa9/2 growth curve on MLA, ½ SSM, BG11, and BG11 TIC 0.00657 M

The ½ SS medium, with the same total ion concentration as MLA, showed good growth rate. It was close to MLA in experiment 3, with a growth rate of 0.14/day. A surprising result came from BG11 TIC 0.00657 M. It reached 3.4×10^6 cells/mL, with a growth rate of 0.19/day. This number was higher than MLA and BG11 in its original TIC in the previous experiment. The contrast between BG11 and BG11 TIC 0.00657 M was revealed not only by the cell number, but also the culture medium colour. In experiment 3, the BG11 colour was pale bright green, and stopped changing on day 4, while in this experiment, the BG11 TIC 0.00657 M colour changed, becoming greener every day. Although the BG11 TIC 0.00657 showed good growth, the difference between its duplicates was a significant problem in this experiment. It would be useful to investigate the effect of total ionic concentration in any following experiment.

The ½ SS medium performed precipitation after sterilization using an autoclave. Precipitation disturbed the cell view under the microscope. In this experiment, the ½ SS medium was diluted to 0.00657 M, and the precipitation faded due to the dilution. The colour of the ½ SS medium with total ionic concentration 0.00657 M stopped changing after day 8.

Similar to the previous experiment, the challenge in this experiment was evaporation. Another difficulty was the precipitation that occurred after culture media sterilization.

Experiment 5 (18 March 2017)

The fifth experiment was performed in order to examine the effect of different total ionic concentrations on cell growth. BG11 (total ionic concentration 0.00657 M and 0.00112 M) and BBM (initial total ionic concentration 0.00657 M) were used in this experiment. The experiment was performed at room temperature with a light intensity of 60 µmol photons/m²s, and 150 RPM. The strain used to start the cultivation was taken from a 37 day old culture in BG11 medium total ionic concentration 0.00657 M with a cell density of 4.885×10^6 cells/mL. A sample of 0.5 mL was taken from each flask every day, using a 1000 µL micropipette. The time span between sampling and cell counting was the same as in previous experiments.

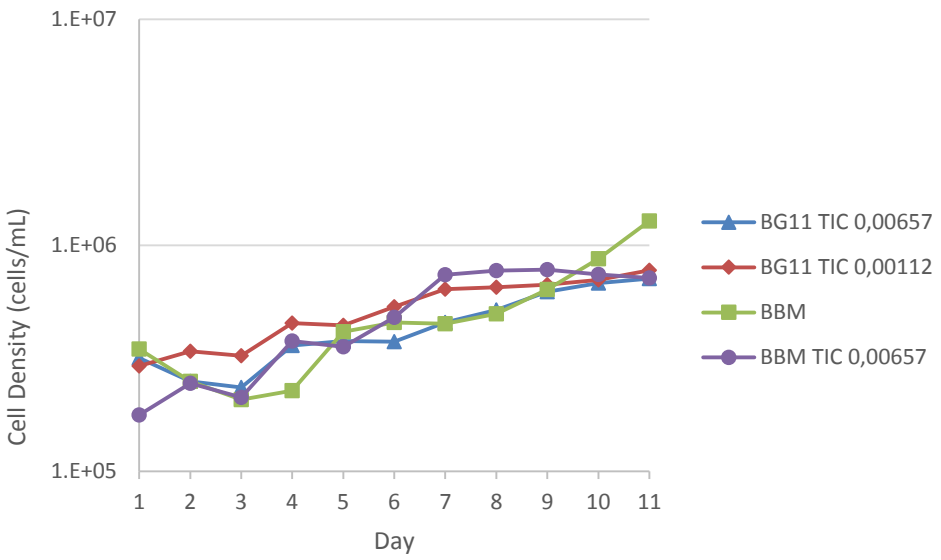

Figure 87. *T.* sp. LCR-Awa9/2 growth curve on BG11 TIC 0.00112, BG11 TIC 0.00657, BBM, and BBM TIC 0.00657

The growth of both BG11 and BBM in various TIC was lower than that of the previous experiments. The effect of different TIC could not be seen clearly in the growth curve. In the first three days, the cells numbers decreased and started to rise again after day 4. The BG11 with TIC 0.00657 in Experiment 4 could reach 1.6×10^6 cells/ml on day 11 with a growth rate of 0.19/day while in this experiment, it only produced 7.1×10^5 cells/ml with a growth rate of 0.06/day.

The growth rate of BG11 TIC 0.00657 M, BG11 TIC 0.00112 M, BBM, and BBM TIC 0.00657 M was 0.06/day, 0.07/day, 0.09/day, and 0.1/day respectively. The low growth rate was caused by the starter strain used for the cultivation going 37 days old and may have been in its death phase. Evaporation was still a concern.

Experiment 6 (29 March 2017)

Experiment 6 was done by growing the microalgae on eight culture media, OHM sterilized by autoclave, OHM sterilized by filtration, Svetoslav medium, MLA medium without vitamin, BG11 TIC 0.00657, BBM, ½ SSM, and MLA. The cultivation was at room temperature with a light intensity of 60 µmol photons/m²s, and at 150 RPM. The sampling was done using 4 flasks at one time, and the cell counting was done at the SP Lab. A 0.5 mL sample was taken from each flask every day using a 1000 µL micropipette.

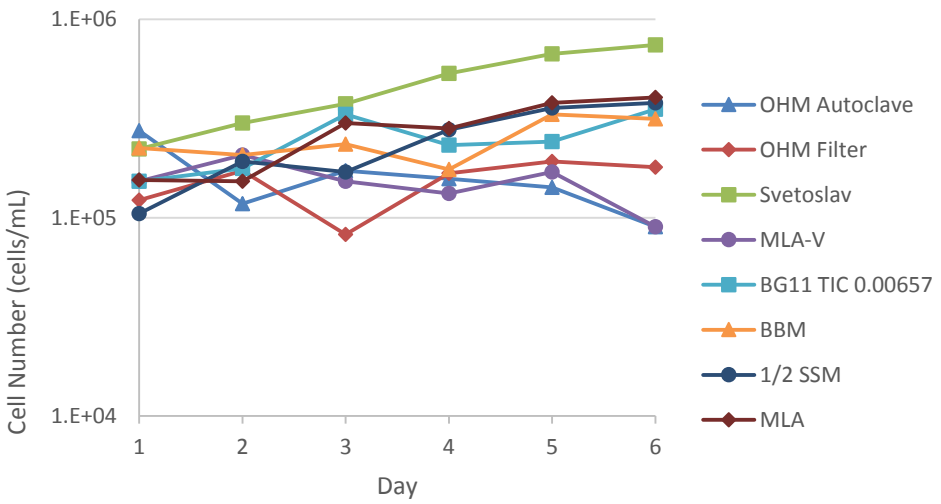

Figure 88. *T.* sp. LCR-Awa9/2 growth curve on OHM sterilized by autoclave, OHM sterilized by filter, Svetoslav medium, MLA medium without vitamin, BG11 TIC 0.00657, BBM, ½ SSM, and MLA

The OHM sterilized by autoclave and filtration did not indicate any significant difference. Both OHM and MLA without vitamin did not show any growth improvement. Their cell number decreased day by day. Both of them were made without vitamin. Other compositions followed the original recipe, except MLA (selenium acid was replaced by selenium dioxide).

BG11 and BBM showed an insignificant rise in cell number, but was inconsistent, while ½ SSM and MLA showed a similar pattern as in the previous experiment although the growth rate was lower than before. Only the Svetoslav medium performed well in this experiment.

The experiment was stopped at day 6 because of the data chaos. The time span between sampling and cell counting was too long, resulting in difficulty with cell counting. The cells in the sample settled at the bottom of the tubes which caused them to become colonized even after homogenization as written in Appendix C Section Sampling and Cell Counting. Like previous experiments, evaporation due to uncontrolled temperature still occurred.

Based on the condition of the experiment, if cultivation of more than four flasks was performed, and the places for sampling and cell counting were at a distance from one another, it was not recommended to do more than two samplings at one time.

From the first to the sixth experiment, cell counting at day zero, right after strain transferring, was not done. This is why some cell number data on the first and second day were lower than the expected original cell number, which was 2×10^5 cells/mL. Day zero cell counting is needed to ensure that the microalgae environment was started with the correct number of cells.

Experiment 7 (2 May 2017)

Experiment 7 was completed by cultivating the microalgae on Svetoslav medium and ½ SS medium in duplicates. The Svetoslav medium was sterilized by filtration and the MLA medium without vitamin was put on trial as well.

The cultivation was performed at room temperature with a light intensity of 60 µmol photons/m²s and at 150 RPM. The sampling was done with one sample at a time and the time span between sampling and cell counting was much faster because the microscope was moved closer to the laminar flow. This made it possible to do one sampling at a time, not multiple samples at one time as in previous experiments. Day zero cell counting was done to confirm that the initial cell number was 2×10^5 cells/mL. The sampling was done with three flasks at one time with a 0.5 mL sample taken every day, using a 1000 µL micropipette.

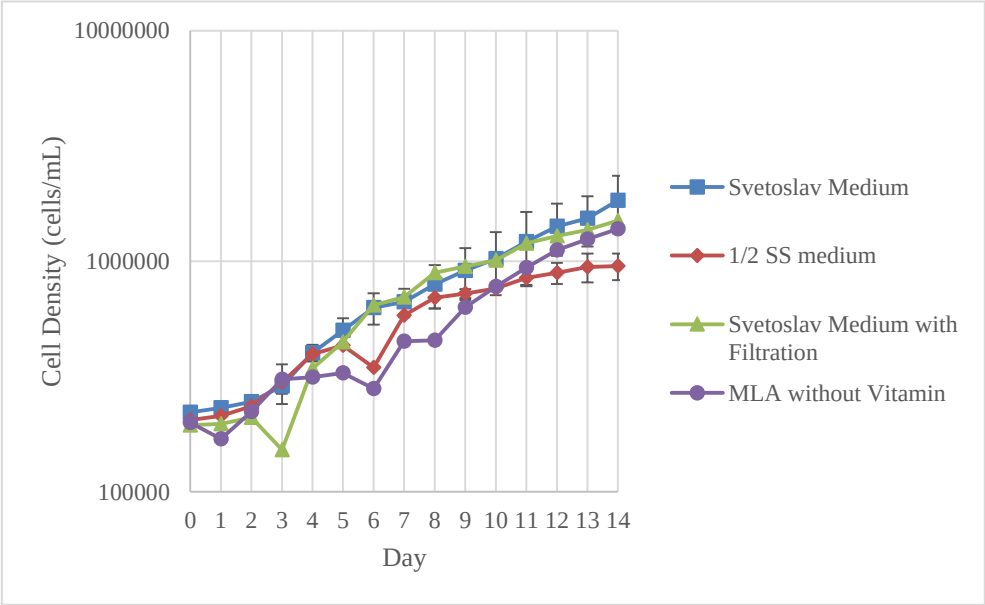

Figure 89. *T.* sp. LCR-Awa9/2 growth curve on Svetoslav medium sterilized by autoclave (in duplicates), ½ SS medium (in duplicates), Svetoslav medium sterilized by a filter, MLA medium without vitamin.

The cultivation was confirmed as starting with no less than 2×10^5 cells/mL except the Svetoslav medium sterilized by a filter (started with 1.952×10^5 cells/mL). The Svetoslav medium sterilized by autoclave or filter did not present significant differences, as shown by OHM in the previous experiment.

The average differences between duplicates of the Svetoslav medium and ½ SS medium were 17% and 7% respectively. The growth rates of ½ SSM and Svetoslav medium were similar until day 4, then the ½ SS medium became slower. There were three drops in the Svetoslav medium sterilized by a filter (day 3), the MLA medium without vitamin (day 6), and the ½ SS medium (day 6). This could be caused by an error in cell counting, even in the ½ SS medium, which was done in duplicates. A possible solution for this problem is that if any drop is found after cell counting, it is better to do the sampling one more time directly after the first sampling to confirm the previous result.

An unexpected result came from MLA without vitamin. It worked well in this experiment. This MLA medium without vitamin was made by removing selenium from its recipe. The growth rate was 0.16/day, faster than the ½ SS medium with a growth rate of 0.12/day.

Even though time span was not a problem in this experiment, evaporation still occurred.

After Experiment 6 and 7, it was decided that the Svetoslav medium would be used to maintain fresh cell stocks for future experiments.

Experiment 8 (16 May 2017)

In the eighth experiment, cultivation was performed in the MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers incubator with temperature control and a higher light intensity than the OM11 medium Orbital Shaker incubator. The temperature was set at 25 ˚C at medium light intensity, 150 RPM. In this experiment, Svetoslav medium, BG11 medium, BBM, and ½ SS medium were used for the microalgae cultivation, each in duplicates. The aim of this experiment was to test the new incubator performance.

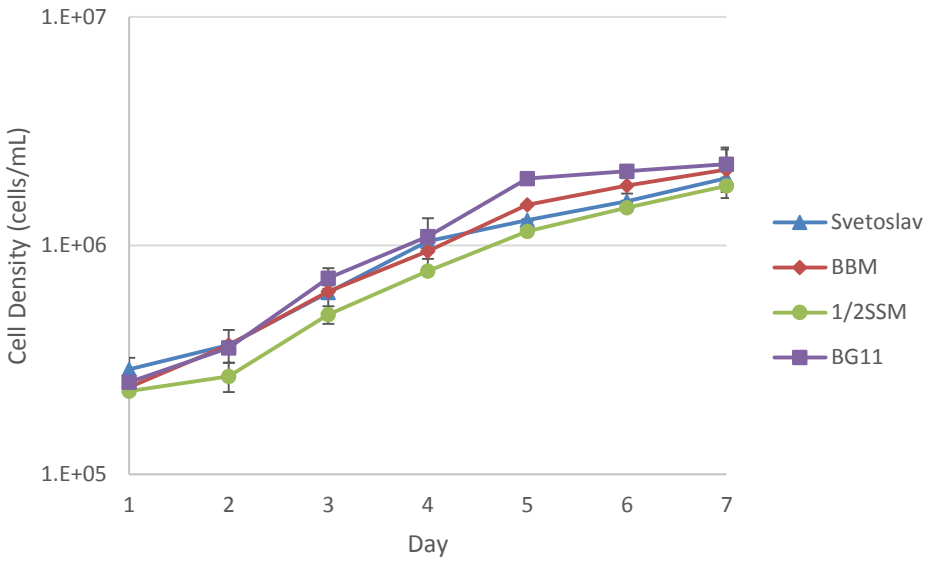

Figure 90. *T.* sp. LCR-Awa9/2 growth curve in Svetoslav medium, ½ SS medium, BG11 medium, and BBM, all were in duplicates.

A remarkable improvement was gained in this experiment. By using the new incubator, the growth rates were twice as fast as cultivation using the old incubator. The improvements were in light intensity, temperature controlled, and a closed system.

Using the previous incubator, cultivation in Svetoslav medium in day 7 gained 6.6×10^5 cells/mL while in the new incubator, the cell density was up to 1.9×10^6 cells/mL. The ½ SS medium also improved from 5.8×10^5 cells/mL to 1.8×10^6 cells/mL using the new incubator. This proved that light intensity along with stable temperature significantly affected the growth rate.

Another surprising results came from BG11 and BBM. Both of these culture media had not been successful in the old incubator, but performed satisfactorily in this experiment. This needed further investigation as soon as the gas system was set on the new incubator.

The day 0 cell density of 8 flasks was counted, but gave unusual results. Most of them were below 1×10^5 cells/mL in the second count, which was impossible because the first results

showed that the cell densities were averaging 1.7×10^5 cells/mL. A few strains were added to achieve 2×10^5 cells/mL as a starting point. Therefore, the second cell count after the second strain transfer should have been higher than the first. This was caused by the strain transferring, and as the cell counting for the 8 flasks was done at a time, some cell sedimentation may have occured. For the next experiment, it is suggested that the strain transfer be done one flask at a time. The starter strain should then be put in the incubator after each transfer. The sequence should be, first, transfer some amount of starter to flask A, put the starter cells back into the incubator while counting flask A day 0 cell density, then take the starter cells from the incubator to the laminar flow again to start flask B, and so forth.

Even though the temperature was controlled at 25 ˚C, some evaporation still occurred in this experiment. An experiment needs to be conducted in order to discover the degree of water loss during the cultivation. The evaporation was suspected as the reason why the growth curve did not reach the death phase, but kept increasing day after day, even after 30 days.

Experiment 9 (3 June 2017)

The purpose of experiment 9 was to make T. sp. LCR-Awa9/2 growth curve using 100 mL Svetoslav medium in each flasks. Two flasks were prepared to cultivate the algae with an initial cell number of 3×10^5 cells/mL and another one with an initial cell number of 2×10^5 cells/mL. The three flasks were kept in the incubator, at 25 °C, 150 RPM, and medium light intensity.

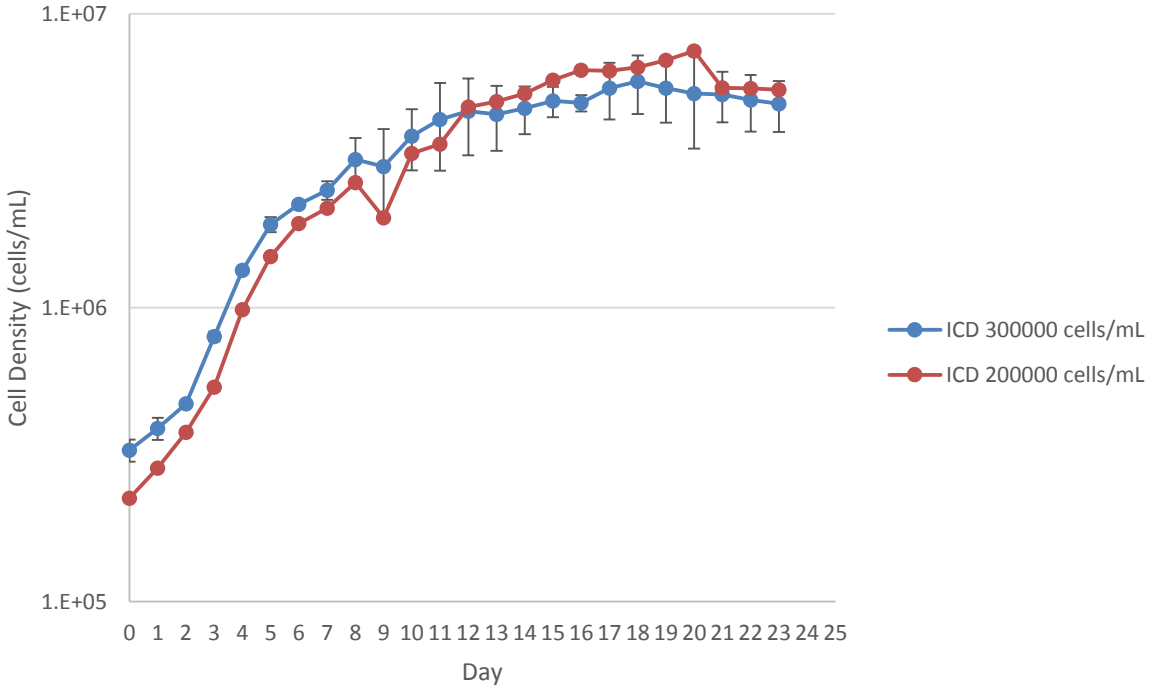

Figure 91. *T.* sp. LCR-Awa9/2 growth curve in Svetoslav medium with different initial cell densities (ICD)

As a confirmation for Experiment 8, the new incubator gave a significant improvement on the growth curve. The culture reached 1×10^6 cells/mL in day 4 while it took 10 days to reach that number using the old incubator.

Until Experiment 9, the cultivation did not reach the death phase. The cell density kept increasing day after day even until day 30. Evaporation was suspected to be the cause of the problem. The old incubator did not have a proper temperature control, and the temperature during the experiment was up to 30 °C. In this experiment, the cultures entered their stationary phase in day 12 and started to decrease after day 20.

The culture that started with 3×10^5 cells/mL did not show significant difference with the one with an initial density of 2×10^5 cells/mL. The difference between duplicates during the exponential phase was relatively low (lower than 10%), but after entering to the stationary phase, the error percentage increased and could be up to 30%.

Experiment 10 (3 June 2017)

The aim of this experiment was to try the universal Zehnder medium. 100 mL of the culture media was prepared in duplicates. The cultivation was performed at 25 ˚C, 150 RPM, and medium light intensity.

The sample was taken daily. In the first 4 days, a 0.5 mL sample was taken using a 1000 µL micropipette. Afterward, a 20 µL sample was taken and diluted with 180 µL distilled water, resulting in a dilution factor of 10. This was conducted to reduce the risk of error while counting cells. Without dilution, counting cells when the density was more than 1×10^6 was time consuming work and ran the risk of human error. Additionally, taking 0.5 mL for sampling was too much as it would affect the change of volume resulting in the growth curve.

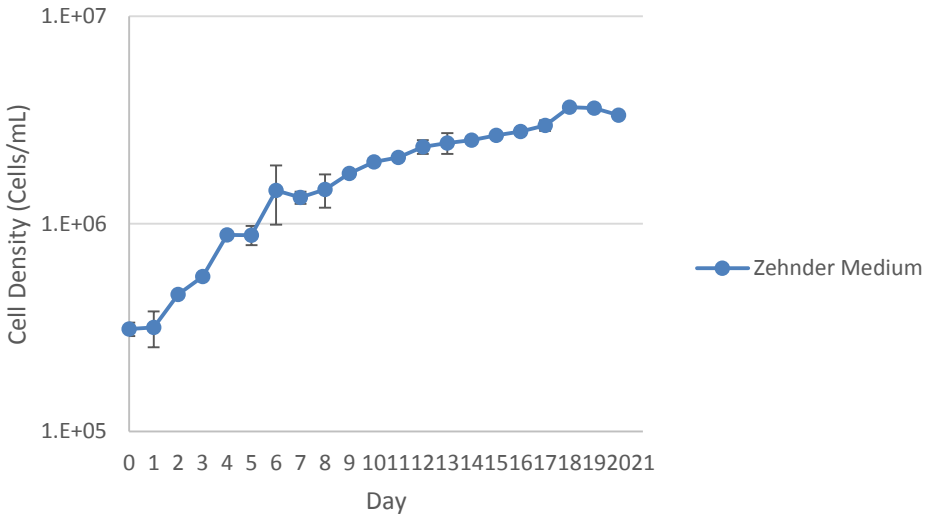

Figure 92. *T.* sp. LCR-Awa9/2 growth curve on Zehnder medium.

The growth of the microalgae in Zehnder medium (ZM) was pleasing, but the result could not compete with the Svetoslav medium. In Experiment 8, the Svetoslav medium reached 1×10^6 cells/mL at day 5 while the Zehnder medium was a day slower. The growth rate gained from this experiment was 0.12/day with a duplicate difference less than 20% (except day 6 with an error of 32%). As an improvement on Experiment 8, transferring the starter strain in this experiment was done one flask at a time. After the cells were transferred to flask A, the starter strain flask was put back in the incubator while Flask A day zero cell density was counted. Then, the starter flask was brought back to the laminar flow and a sample was taken from it to be transferred to Flask B. Finally, the starter flask was carried back to the incubator and Flask B day 0 cell density was calculated.

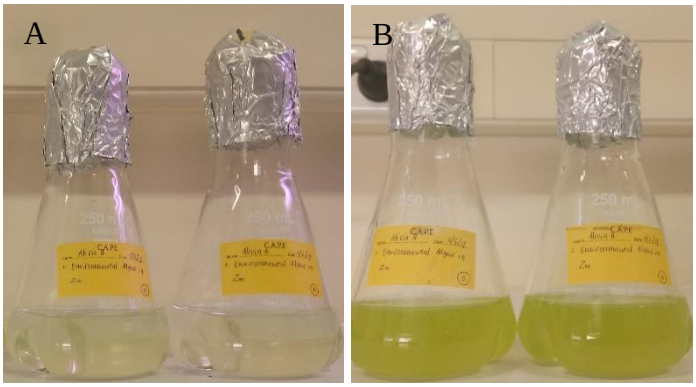

Figure 93. Environmental Algae in Zehnder medium (A. Day 0; B. Day 14)

Experiment 11 (31 May 2017)

The practical aim of Experiment 10 was to determine the cell counting error was gained during the experiment. An environmental alga was prepared using Svetoslav medium with a starter cell density of 2×10^5 cells/mL. It was cultivated in the incubator at 25 ˚C, 150 RPM, and medium light intensity. In the first 5 days, a 0.5 mL sample was taken from the flask. Starting from day 6, 20 µL of sample was taken and diluted to 180 µL sterile water, resulting in a dilution factor of 10. The sampling was conducted three times a day in sequence (the time span between each sampling was less than 10 minutes).

To prevent the cells became settled at the bottom of the flask, the environmental algae was returned to the incubator directly after each sampling. After some volume of sample was taken from the flask, it was restored to the shaker incubator while the first sampling cell density was counted. Then the flask was brought back to the laminar flow for the second sampling, and so forth.

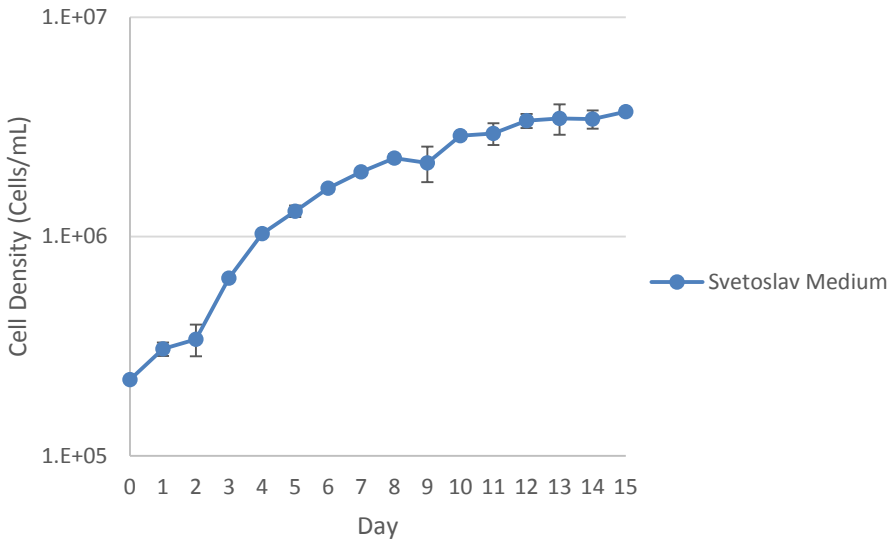

Figure 94. Environmental Algae in Svetoslav medium

Table 21. Experiment 11 cell density and error percentage

Day	1st Sampling	2nd Sampling	3rd Sampling	Average	Standard Deviation	Standard Error
0	222500	-	-	-	-	-
1	282500	312500	325000	306666.7	21250	7%
2	372500	267500	380000	340000	56250	17%
3	675000	627500	632500	645000	23750	4%
4	1042500	1017500	1030000	1030000	12500	1%
5	1242500	1272500	1397500	1304167	77500	6%
6	1722500	1657500	1600000	1660000	61250	4%
7	1912500	1947500	2045000	1968333	66250	3%
8	2295000	2170000	2367500	2277500	98750	4%
9	1650000	2450000	2400000	2166667	400000	18%
10	3000000	2800000	2850000	2883333	100000	3%
11	2875000	3325000	2650000	2950000	337500	11%
12	3150000	3650000	3325000	3375000	250000	7%
13	3325000	2975000	4075000	3458333	550000	16%
14	3425000	3100000	3750000	3425000	325000	9%
15	3850000	3700000	3570000	3706667	140000	4%

Table 22. shows that the maximum error was 18% which happened in day 9. Overall, the cell counting errors were below 18%. This would became a reference of the cell counting accuracy which was performed by the researcher.

Experiment 12 (12 June 2017)

There were two issues which had not been resolved from previous experiments namely, evaporation and death phase. The death phase did not occur which could have been caused by

the change of volume. Evaporation and the amount of sample taken were the main cause. In this experiment, volume loss during the cultivation process was monitored and the amount of sample taken for cell counting was reduced.

The test used the Svetoslav medium (sterilized by filtration) with an initial cell density of 2.6×10^5 cells/mL at 25 ˚C, 150 RPM, and medium light intensity.

The initial volume of the culture was 106 mL and no samples were taken until day 16. The sampling was done only at day 0 to make sure that the initial cell density was at least 2×10^5 cells/mL, and in day 16 where the final volume was measured. The final volume was 102 mL, where 0.2 mL were taken for 2 sampling as mentioned before. In a cultivation period of 16 day using new incubator, there was approximately 4 mL volume loss. The loss had to be higher when using the old incubator since it has poor temperature controller.

The final cell density was 2×10^6 cells/mL which was lower than the growth in Experiment 11 and 9 in day 16.

Experiment 13 (7 July 2017)

The practical aim of Experiment 13 was to make sure that the modified MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers worked well. After adjusting the length of the stoppers' pipe to be 0.5 cm below 100 mL liquid surface, this trial meant to ensure that the gas was evenly distributed in all 18 flasks. In a flow rate of 100 cm^3/min, there was approximately 10 bubbles per 7 seconds in each flask.

Four 250 mL baffled flask was prepared, each contained 100 mL of culture media, 2 flasks of Svetoslav medium and 2 flasks of Zehnder medium. A 27 mL of inoculum (9.45×10^5 cells/mL) was transferred into each flask, resulted in a total volume of 127 mL. Due to volume increase, the flow rate was increased to 400 cm^3/min (3% CO_2 enriched air) to get at least 1 bubble per second. The cultures were incubated at 25 °C, high light intensity, and shaken at 150 RPM.

A cell count was done immediately after inoculum transfer into each flask to ensure that each flask start with an initial cell density of 2×10^5 cells/mL.

In day 1, the evaporation happened severely. This was caused by the high light intensity. When the incubator door opened, a bare hand could feel the warm atmosphere inside the incubator. Even the temperature control could not prevent the evaporation which was triggered by warm light. The light intensity was set at level 4 because there were many tubes hanging around the incubator and there were 18 flasks in the incubator so shadows might occur. The light intensity then reduced to medium light intensity.

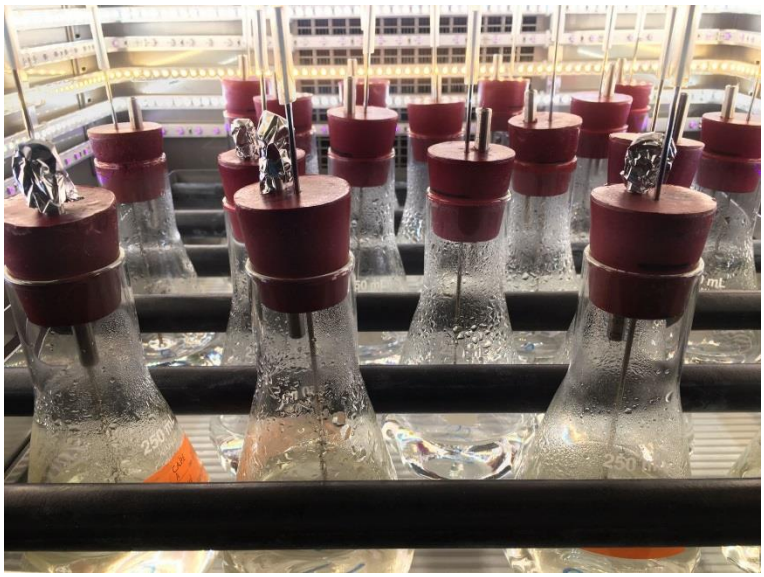

Figure 95. Evaporation in the first day of cultivation

It was necessary to follow the sampling procedure. Taking sample from the flask and injecting it directly into the hemacytometer was not effective. The error between repetitions was high. A proper sample homogenization is essential in sampling and cell counting.

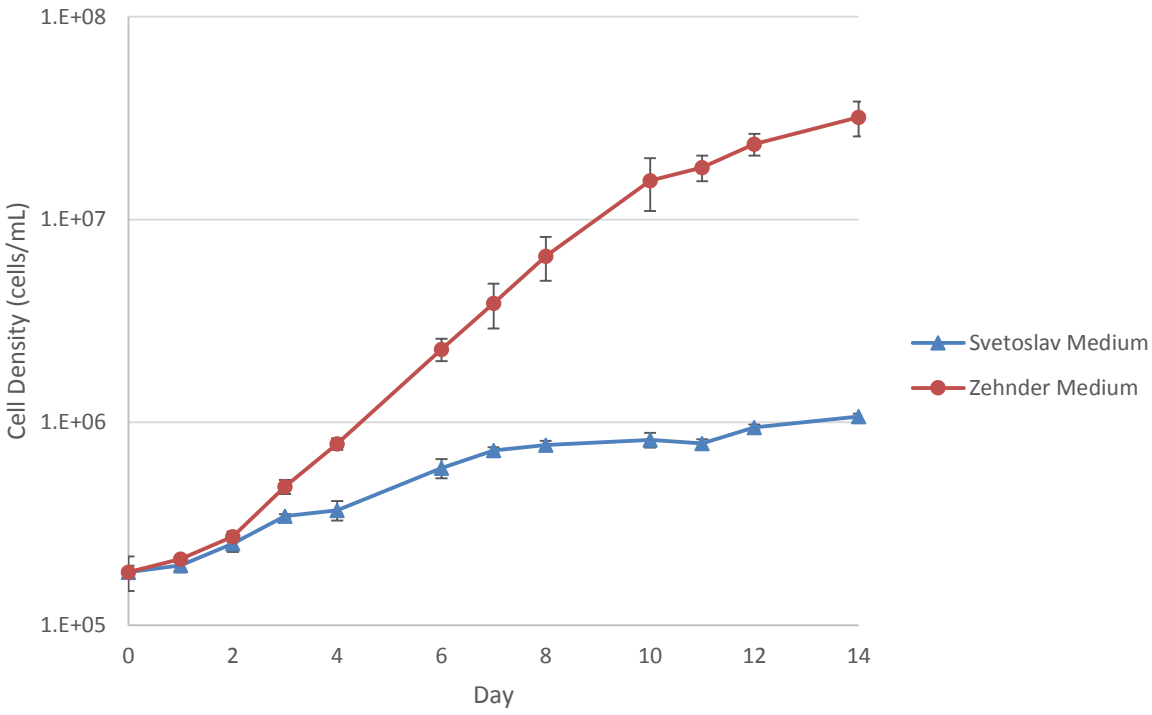

Figure 96. *T.* sp. LCR-Awa9/2 growth curve in Svetoslav and Zehnder medium

In the first 2 days, both culture media performed similar growth rate. In the 3rd day, Zehnder medium enter its exponential phase while Svetoslav medium only increased slightly. This was the opposite of Experiment 9 and 10, where Svetoslav perform faster growth rate than Zehnder medium. This showed that sparging CO_2 gave significant improvement on cultivation process.

Svetoslav medium and Zehnder Medium had same micronutrient chemicals but different concentration for each chemical. The differences between Zehnder Medium and Svetoslav medium was in the macronutrient components. Svetoslav medium used sodium bicarbonate instead of sodium carbonate. It contained sodium chloride while Zehnder medium did not use it. As the source of iron, Svetoslav medium used ferrous sulphate whereas Zehnder medium used ferric EDTA. They also had different total ionic concentration. The TIC of Zehnder medium was 0.0133 M while the TIC of Svetoslav medium was 0.0593 M, 4.5 times higher than Zehnder medium.

The initial pH of Svetoslav and Zehnder medium was 8.89 and 7.72, respectively. In the end of the experiment, the pH of Svetoslav culture in flask A and B was change into 6.77 and 6.73, respectively. The final pH of Zehnder culture in flask C was 7.49, and in flask D 9.32.

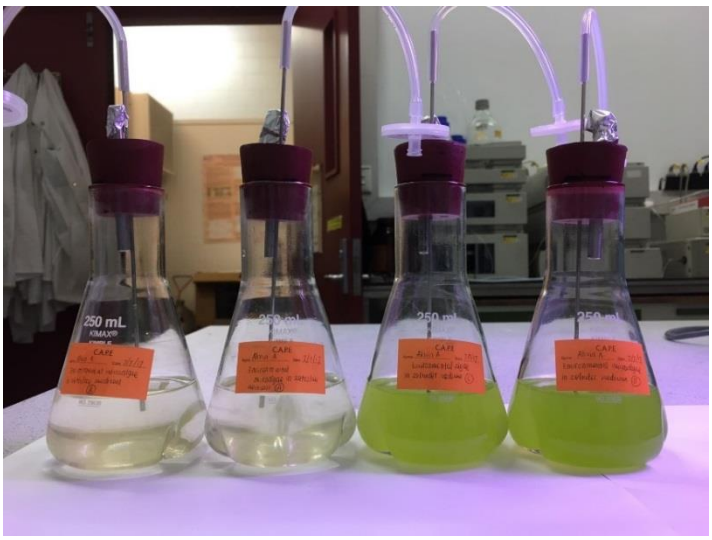

Figure 97. Experiment 13 environmental algae in day 7

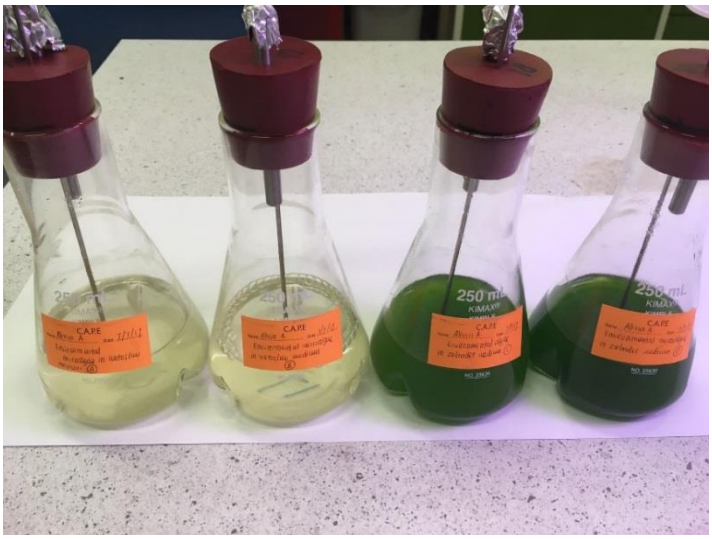

Figure 98. Experiment 13 environmental algae in day 10

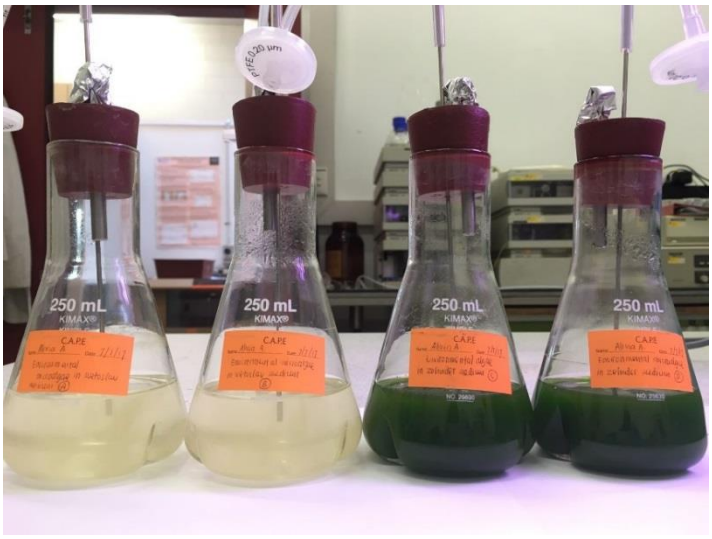

Figure 99. Experiment 13 environmental algae in day 14

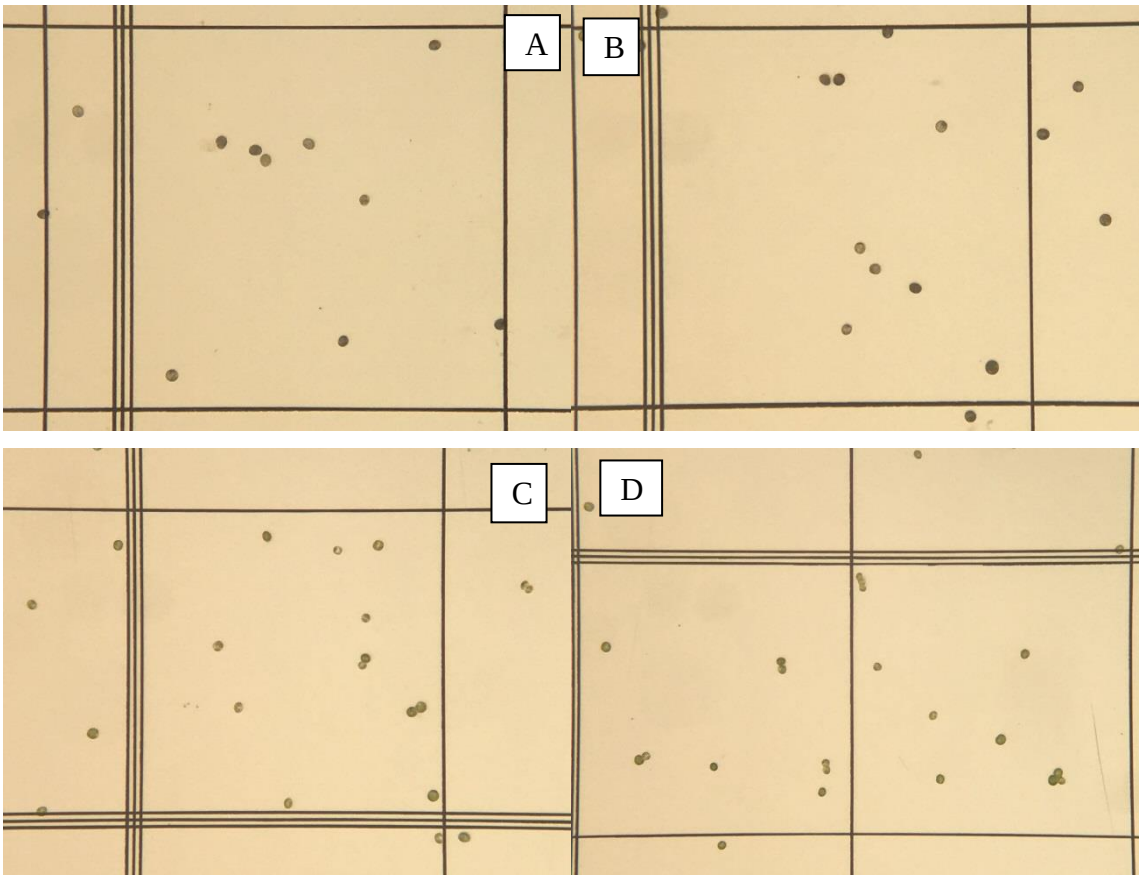

Figure 100. A, B, C, and D were picture of 10 days cells in Svetoslav A, B, Zehnder A, B respectively.

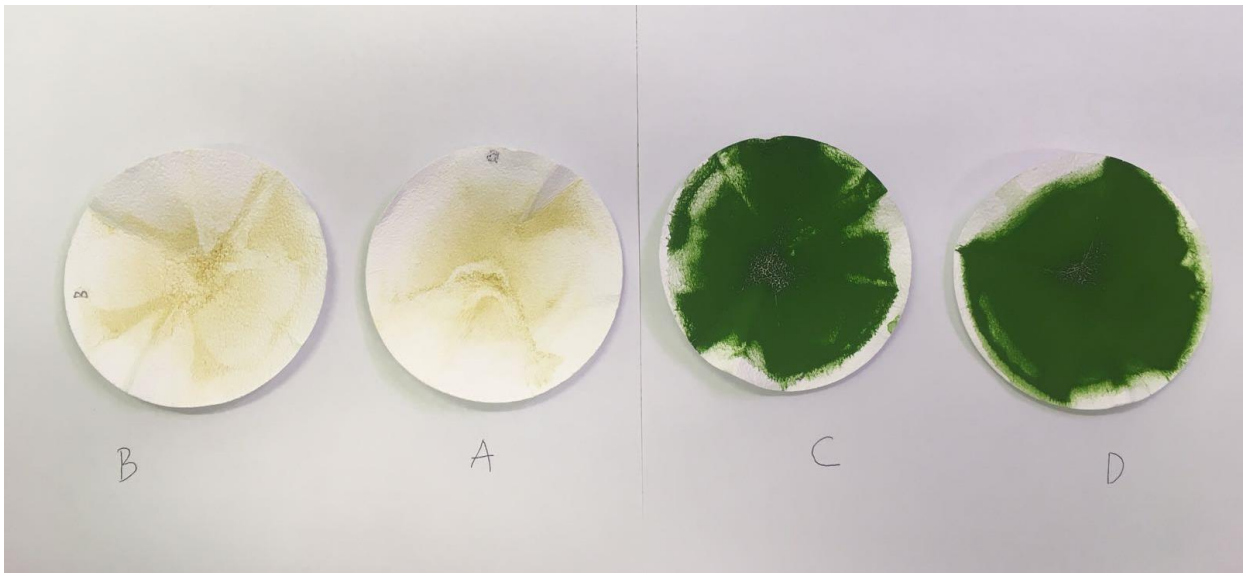

Figure 101. Dried cells (A and B was from Svetoslav medium, C and D was from Zehnder medium)

The biomass gained from this experiment was 0.194, 0.208, 2.468, and 2.56 g/L from flask A, B, C, and D, respectively.

APPENDIX D

EXPERIMENTAL DATA

Alphabets : The flask code
SD : Standard deviation
SE : Standard error

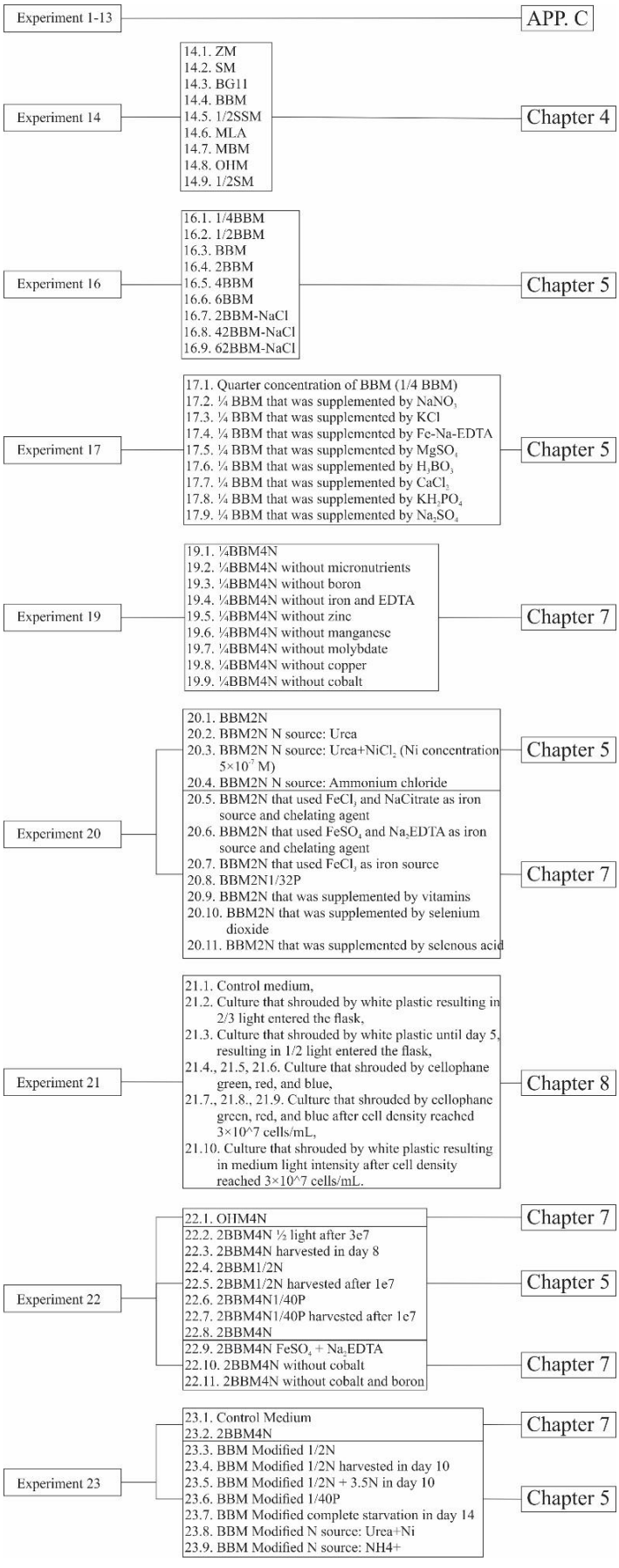

Figure 102. Thesis layout

Experiment 14

Experimental conditions:
14.1. ZM
14.2. SM
14.3 BG11
14.4. BBM
14.5. 1/2SSM
14.6. MLA
14.7. MBM
14.8. OHM
14.9. 1/2SM

Table 22. Experiment 14 cell density.

Day	14.1. Zehnder Medium					14.2. Svetoslav Alexandrov Medium				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	207500	193750	200625	9722	5%	223750	200000	211875	16793	8%
1	223750	221250	222500	1767	1%	200000	166250	183125	23864	13%
2	240000	286250	263125	32703	12%	223750	217500	220625	4419	2%
3	511250	448750	480000	44194	9%	366250	332500	349375	23864	7%
4	867500	791250	829375	53916	7%	415700	545000	480350	91428	19%
5	1575000	1150000	1362500	300520	22%	662500	641250	651875	15026	2%
6	2537500	1750000	2143750	556846	26%	688750	787500	738125	69826	9%
7	5012500	4250000	4631250	539168	12%	927500	1052500	990000	88388	9%
8	6950000	6725000	6837500	159099	2%	1076250	1206250	1141250	91923	8%
9	10500000	10175000	10337500	229809	2%	1300000	1350000	1325000	35355	3%
10	14425000	12025000	13225000	1697056	13%	1325000	1362500	1343750	26516	2%
11	17250000	14900000	16075000	1661701	10%	1862500	1325000	1593750	380069	24%
12	21350000	19450000	20400000	1343503	7%	1725000	1200000	1462500	371231	25%
13	22050000	24900000	23475000	2015254	9%	2337500	1992500	2165000	243951	11%
14	23250000	27750000	25500000	3181981	12%	2637500	2225000	2431250	291681	12%

Day	14.9. 1/4 Svetoslav Alexandrov Medium					14.3. Blue Green 11 Medium				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	207500	197500	202500	7071	3%	208750	225000	216875	11490	5%
1	193750	232500	213125	27400	13%	220000	211250	215625	6187	3%
2	223750	291250	257500	47729	19%	248750	262500	255625	9722	4%
3	497500	461250	479375	25632	5%	438750	371250	405000	47729	12%
4	866250	936250	901250	49497	5%	981250	875000	928125	75130	8%
5	1875000	1525000	1700000	247487	15%	1512500	1750000	1631250	167937	10%
6	2537500	3050000	2793750	362392	13%	2150000	2375000	2262500	159099	7%
7	5687500	5350000	5518750	238648	4%	4837500	4700000	4768750	97227	2%
8	6975000	8575000	7775000	1131371	15%	5025000	5025000	5025000	0	0%
9	10525000	10875000	10700000	247487	2%	7700000	6700000	7200000	707106	10%
10	15375000	13525000	14450000	1308148	9%	8775000	10225000	9500000	1025305	11%
11	17600000	16700000	17150000	636396	4%	11900000	11200000	11550000	494974	4%
12	17050000	20000000	18525000	2085965	11%	14150000	17050000	15600000	2050610	13%
13	21050000	23250000	22150000	1555635	7%	18750000	20750000	19750000	1414214	7%
14	22450000	26800000	24625000	3075914	12%	22250000	21250000	21750000	707106	3%

Day	14.4. Bold Basal Medium					14.5. 1/2 Schlender Setlik Medium				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	196250	205000	200625	6187	3%	203750	211250	207500	530	3%
1	193750	221250	207500	19445	9%	242500	210000	226250	22980	10%
2	272500	233750	253125	27400	11%	252500	201250	226875	36239	16%
3	496250	562500	529375	46845	9%	365000	307500	336250	40658	12%
4	1075000	1022500	1048750	37123	4%	586250	621250	603750	24748	4%
5	1587500	1462500	1525000	88388	6%	950000	775000	862500	123743	14%
6	2787500	2512500	2650000	194454	7%	1300000	1175000	1237500	88388	7%
7	5700000	4962500	5331250	521491	10%	2187500	2162500	2175000	17677	1%
8	10400000	8975000	9687500	1007627	10%	3375000	3475000	3425000	70710	2%
9	13800000	14075000	13937500	194454	1%	4300000	3825000	4062500	335875	8%
10	17000000	17550000	17275000	388908	2%	6850000	6875000	6862500	17677	0%
11	18550000	20950000	19750000	1697056	9%	8500000	7750000	8125000	530330	7%
12	24300000	26500000	25400000	1555635	6%	8750000	7900000	8325000	601040	7%
13	27500000	28950000	28225000	1025305	4%	14250000	8725000	11487500	3906765	34%
14	31050000	33300000	3.22E+07	1590990	5%	19450000	8000000	13725000	8096373	59%
Day	14.6. MLA Medium					14.6. Modified Bourrelly Medium				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	190000	202500	196250	8838	5%	192500	183750	188125	6187	3%
1	176250	196250	186250	14142	8%	210000	238750	224375	20329	9%
2	260000	218750	239375	29168	12%	257500	267250	262375	6894	3%
3	206250	147500	176875	41542	23%	455000	516250	485625	43310	9%
4	257500	196250	226875	43310	19%	777500	886250	831875	76897	9%
5	230000	216250	223125	9722	4%	1412500	1300000	1356250	79549	6%
6	260000	238750	249375	15026	6%	2112500	1862500	1987500	176776	9%
7	383750	311250	347500	51265	15%	3812500	3612500	3712500	141421	4%
8	515000	492500	503750	15909	3%	6225000	5975000	6100000	176776	3%
9	745000	712500	728750	22980	3%	8425000	8800000	8612500	265165	3%
10	1177500	1062500	1120000	81317	7%	14125000	11625000	12875000	1767767	14%
11	1875000	1625000	1750000	176776	10%	19100000	17200000	18150000	1343503	7%
12	3450000	3225000	3337500	159099	5%	22450000	20950000	21700000	1060660	5%
13	5250000	6025000	5637500	548007	10%	26350000	25050000	25700000	919238	4%
14	7050000	7050000	7050000	0	0%	31800000	32800000	32300000	707106	2%

Day	14.7. Optimum Haematococcus Medium				
	Q	R	Average	SD	SE
0	183750	187500	185625	2651	1%
1	218750	246250	232500	19445	8%
2	240000	268750	254375	20329	8%
3	391250	467500	429375	53916	13%
4	1040000	1050000	1045000	7071	1%
5	1837500	2012500	1925000	123743	6%
6	3337500	3325000	3331250	8838	0%
7	6425000	7075000	6750000	459619	7%
8	8450000	10475000	9462500	1431891	15%
9	13175000	13300000	13237500	88388	1%
10	16250000	14550000	15400000	1202082	8%
11	22300000	21250000	21775000	742462	3%
12	25350000	24600000	24975000	530330	2%
13	31750000	30450000	31100000	919238	3%
14	36850000	29900000	3.34E+07	4914392	15%

Table 23. Experiment 14 pH change

Culture	14.1. ZM		14.2. SM		14.3. BG11M		14.4. BBM		14.5. 1/2 SSM		14.6. MLAM		14.7. MBM		14.8. OHM		14.9. 1/4 SM	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Initial pH	7.72		8.89		8.89		7.17		6.37		5.52		8.05		6.51		7.57	
Final pH	7.4	7.32	6.93	6.63	7.42	7.43	7.14	7.1	7.77	7.59	7.45	7.44	7.23	7.25	7.41	7.51	7.17	7.38
Average	7.36		6.78		7.425		7.12		7.68		7.445		7.24		7.46		7.275	
SD	0.0566		0.2121		0.0071		0.0283		0.1273		0.0071		0.0141		0.0707		0.1485	
SE	1%		3%		0%		0%		2%		0%		0%		1%		2%	

Table 24. Experiment 14 biomass dry weight

Culture	14.1. ZM		14.2. SM		14.3. BG11M		14.4. BBM		14.5. 1/2 SSM		14.6. MLAM		14.7. MBM		14.8. OHM		14.9. 1/4 SM	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Mass (g)	2.06	1.968	0.448	0.464	1.644	1.664	2.308	2.396	1.42	1.636	0.712	0.804	2.308	2.108	2.592	2.296	1.852	1.752
Average	2.01		0.46		1.65		2.35		1.53		0.76		2.21		2.44		1.802	
SD	0.0651		0.01		0.01		0.06		0.15		0.07		0.14		0.21		0.0707	
SE	3%		2%		1%		3%		10%		9%		6%		9%		4%	

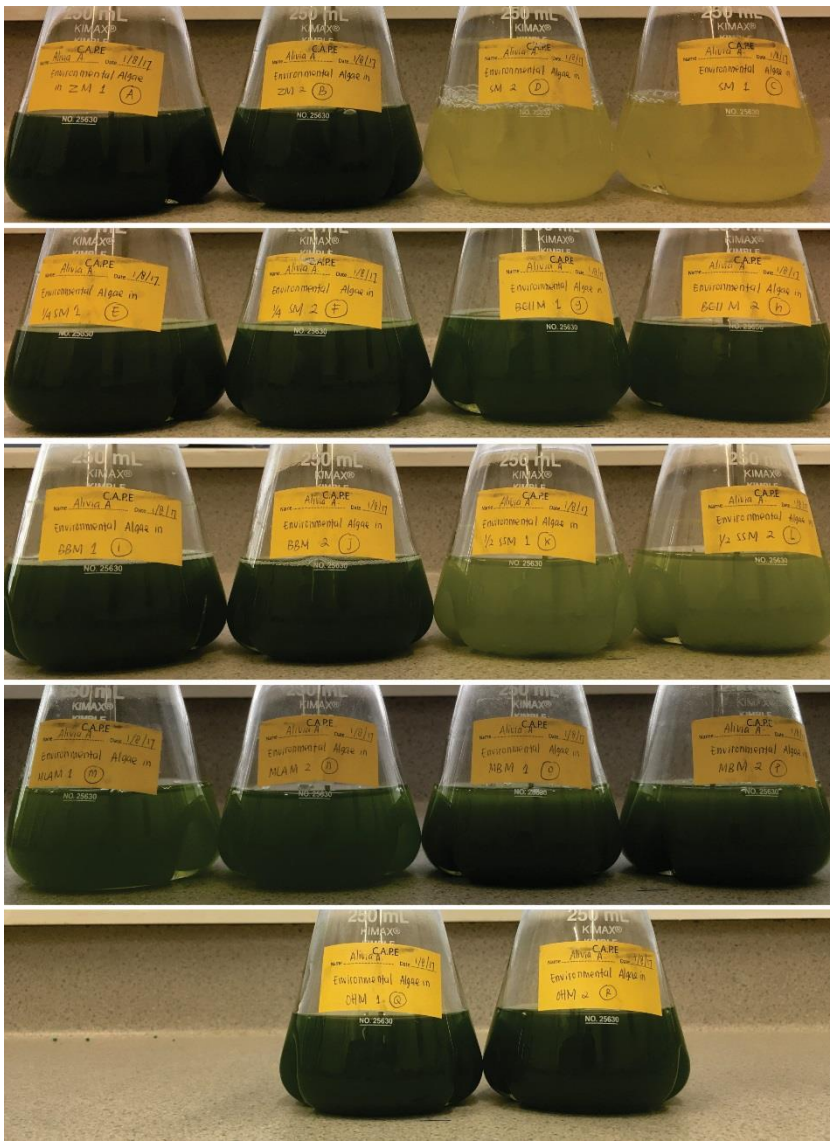

Figure 103. Experiment 14 cultures in day 14.

Experiment 16

Experimental conditions:
16.1. 1/4BBM
16.2. 1/2BBM
16.3. BBM
16.4. 2BBM
16.5. 4BBM
16.6. 6BBM
16.7. 2BBM-NaCl
16.8. 42BBM-NaCl
16.9. 62BBM-NaCl

Table 25. Experiment 16 cell density.

Day	16.1. 1/4BBM					16.2. 1/2BBM				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	227500	193750	210625	23864	11%	258750	281250	270000	15909	6%
1	451250	381250	416250	49497	12%	465000	483750	474375	13258	3%
2	982500	935000	958750	33587	4%	1017500	1012500	1015000	3535	0%
3	1925000	1550000	1737500	265165	15%	1587500	1725000	1656250	97227	6%
4	2925000	3775000	3350000	601040	18%	3625000	2887500	3256250	521491	16%
5	5250000	5525000	5387500	194454	4%	6475000	5700000	6087500	548007	9%
6	4150000	5500000	4825000	954594	20%	7300000	7750000	7525000	318198	4%
7	5750000	6450000	6100000	494974	8%	11550000	9150000	10350000	1697056	16%
8	6600000	5250000	5925000	954594	16%	11950000	13200000	12575000	883883	7%
9	5850000	7400000	6625000	1096015	17%	15900000	12300000	14100000	2545584	18%
10	6150000	5150000	5650000	707106	13%	15600000	12250000	13925000	2368807	17%
11	6450000	5225000	5837500	866205	15%	16250000	12150000	14200000	2899137	20%
12	6200000	6125000	6162500	53033	1%	13750000	11250000	12500000	1767766	14%
13	7750000	5850000	6800000	1343502	20%	12650000	11900000	12275000	530330	4%
14	9000000	7300000	8150000	1202081	15%	15200000	15700000	15450000	353553	2%

Day	16.3. BBM					16.4. 2BBM				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	247500	237500	242500	7071	3%	250000	291250	270625	2916	11%
1	438750	518750	478750	56568	12%	428750	526250	477500	68942	14%
2	985000	1067500	1026250	58336	6%	1132500	1157500	1145000	17677	2%
3	1687500	1687500	1687500	0	0%	1837500	1575000	1706250	185615	11%
4	3250000	2837500	3043750	291681	10%	3500000	2750000	3125000	530330	17%
5	4950000	5300000	5125000	247487	5%	6175000	5725000	5950000	318198	5%
6	7950000	8500000	8225000	388908	5%	10450000	10800000	10625000	247487	2%
7	12850000	12400000	12625000	318198	3%	14900000	17050000	15975000	1520279	10%
8	15900000	17450000	16675000	1096015	7%	17400000	21150000	19275000	2651650	14%
9	18550000	23800000	21175000	3712310	18%	21050000	25000000	23025000	2793071	12%
10	19850000	18850000	19350000	707106	4%	22900000	25400000	24150000	1767766	7%
11	21100000	18950000	20025000	1520279	8%	25050000	28250000	26650000	2262741	8%
12	25700000	19300000	22500000	4525483	20%	27100000	29800000	28450000	1909188	7%
13	24450000	18300000	21375000	4348706	20%	29850000	31850000	30850000	1414213	5%
14	23100000	25050000	24075000	1378858	6%	32300000	39700000	36000000	5232590	15%
Day	16.5. 4BBM					16.6. 6BBM				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	242500	230000	236250	8838	4%	265000	222500	243750	30052	12%
1	472500	481250	476875	6187	1%	466250	515000	490625	34471	7%
2	1060000	1035000	1047500	17677	2%	867500	800000	833750	47729	6%
3	1612500	1875000	1743750	185615	11%	1212500	1337500	1275000	88388	7%
4	2762500	3625000	3193750	609879	19%	1387500	1737500	1562500	247487	16%
5	4775000	4600000	4687500	123743	3%	2150000	2075000	2112500	53033	3%
6	11150000	9900000	10525000	883883	8%	3700000	2850000	3275000	601040	18%
7	13350000	12100000	12725000	883883	7%	3750000	3800000	3775000	35355	1%
8	16900000	17800000	17350000	636396	4%	6050000	5550000	5800000	353553	6%
9	20400000	20200000	20300000	141421	1%	6450000	8600000	7525000	1520280	20%
10	22700000	24150000	23425000	1025305	4%	7900000	7100000	7500000	565685	8%
11	28100000	27800000	27950000	212132	1%	9350000	8800000	9075000	388908	4%
12	28950000	31500000	30225000	1803122	6%	9800000	10150000	9975000	247487	2%
13	29950000	33600000	31775000	2580940	8%	10600000	11750000	11175000	813172	7%
14	36100000	35950000	36025000	106066	0%	11950000	12400000	12175000	318198	3%

Day	16.7. 2BBM-NaCl					16.8. 4BBM-NaCl				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	248750	293750	271250	31819	12%	256250	237500	246875	13258	5%
1	428750	527500	478125	69826	15%	474500	455000	464750	13788	3%
2	1137500	1090000	1113750	33587	3%	1170000	1207500	1188750	26516	2%
3	1600000	2250000	1925000	459619	24%	2087500	2475000	2281250	274003	12%
4	3225000	3362500	3293750	97227	3%	2962500	3025000	2993750	44194	1%
5	6475000	5575000	6025000	636396	11%	5175000	5425000	5300000	176776	3%
6	8400000	10250000	9325000	1308147	14%	8450000	8100000	8275000	247487	3%
7	13100000	12500000	12800000	424264	3%	10300000	12550000	11425000	1590990	14%
8	16000000	15750000	15875000	176776	1%	16750000	15950000	16350000	565685	3%
9	15200000	18100000	16650000	2050609	12%	21100000	17500000	19300000	2545584	13%
10	17100000	22000000	19550000	3464823	18%	23100000	21900000	22500000	848528	4%
11	18400000	24100000	21250000	4030508	19%	24300000	24600000	24450000	212132	1%
12	19750000	25350000	22550000	3959797	18%	25200000	26800000	26000000	1131370	4%
13	20750000	27600000	24175000	4843681	20%	26400000	27100000	26750000	494974	2%
14	24400000	27950000	26175000	2510229	10%	28350000	24900000	26625000	2439518	9%

Day	16.9. 6BBM-NaCl				
	Q	R	Average	SD	SE
0	267500	261250	264375	4419	2%
1	450000	483750	466875	23864	5%
2	1032500	1197500	1115000	116672	10%
3	1912500	2012500	1962500	70710	4%
4	2912500	3575000	3243750	468458	14%
5	5625000	4900000	5262500	512652	10%
6	7500000	7950000	7725000	318198	4%
7	9600000	9750000	9675000	106066	1%
8	13600000	14950000	14275000	954594	7%
9	15600000	17400000	16500000	1272792	8%
10	18500000	19750000	19125000	883883	5%
11	19750000	21400000	20575000	1166726	6%
12	20550000	23200000	21875000	1873832	9%
13	20850000	22650000	21750000	1272792	6%
14	22350000	24550000	23450000	1555634	7%

Table 26. Experiment 16 pH change

Culture	16.1. 1/4 BBM		16.2. 1/2 BBM		16.3. BBM		16.4. 2 BBM		16.5. 4 BBM		16.6. 6 BBM		16.7. 2 BBM NaCl		16.8. 4 BBM NaCl		16.9. 6 BBM NaCl	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Initial pH	6.58		6.54		6.28		6.26		6.16		6.13		6.35		6.19		6.54	
Final pH	7.15	6.78	6.99	6.8	7.29	7.05	7.09	7.31	7.49	7.5	6.85	6.89	7.23	7.36	7.1	7.09	7	7.06
Average	6.965		6.895		7.17		7.2		7.495		6.87		7.295		7.095		7.03	
SD	0.2616		0.1344		0.1697		0.1556		0.0071		0.0283		0.0919		0.0071		0.0424	
SE	4%		2%		2%		2%		0%		0%		1%		0%		1%	

Table 27. Experiment 16 biomass dry weight

Culture	16.1. 1/4 BBM		16.2. 1/2 BBM		16.3. BBM		16.4. 2 BBM		16.5. 4 BBM		16.6. 6 BBM		16.7. 2 BBM NaCl		16.8. 4 BBM NaCl		16.9. 6 BBM NaCl	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Mass (g)	1.144	0.804	1.856	1.812	2.48	2.62	2.88	3.052	2.744	3.156	1.684	1.856	2.68	2.768	2.844	2.628	2.452	2.552
Average	0.97		1.83		2.55		2.97		2.95		1.77		2.72		2.74		2.50	
SD	0.24		0.03		0.10		0.12		0.29		0.12		0.06		0.15		0.07	
SE	25%		2%		4%		4%		10%		7%		2%		6%		3%	

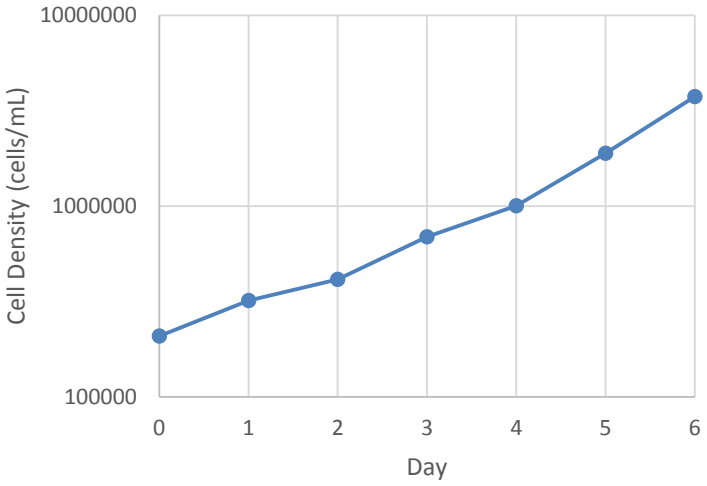

Figure 104. Growth curve of *T.* sp. LCR-Awa9/2 inoculum to start experiment 16.

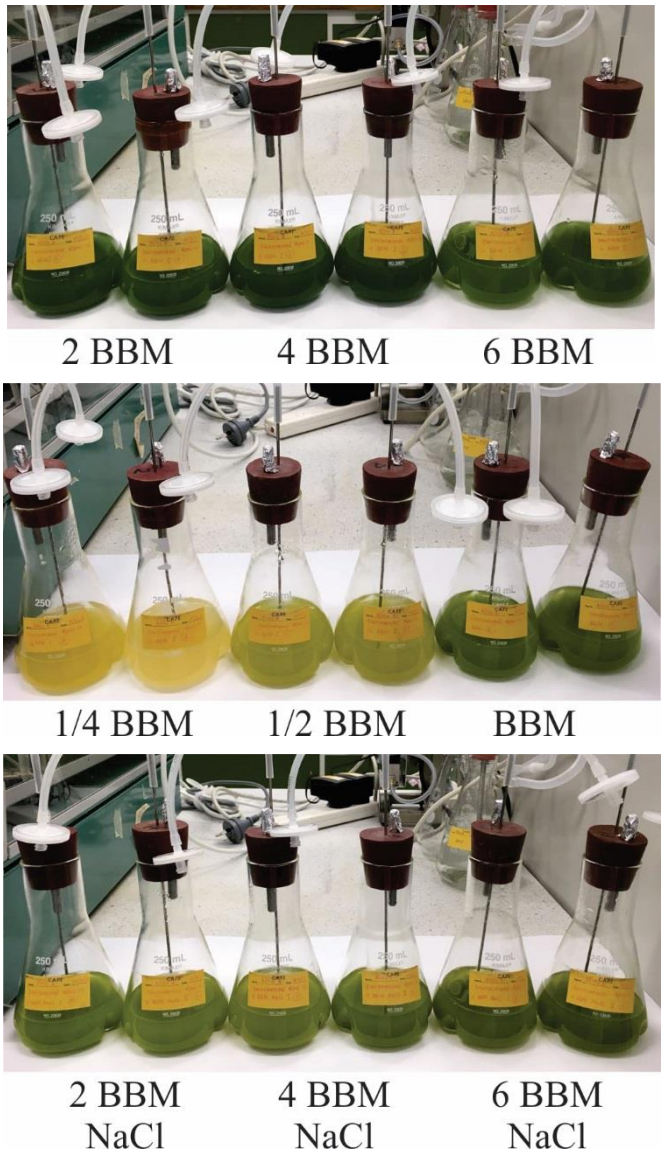

Figure 105. Experiment 16 cultures

Experiment 17

Experimental conditions:
17.1. Quarter concentration of BBM (1/4 BBM)
17.2. ¼ BBM that was supplemented by $NaNO_3$
17.3. ¼ BBM that was supplemented by KCl
17.4. ¼ BBM that was supplemented by Fe-Na-EDTA
17.5. ¼ BBM that was supplemented by $MgSO_4$
17.6. ¼ BBM that was supplemented by H_3BO_3
17.7. ¼ BBM that was supplemented by $CaCl_2$
17.8. ¼ BBM that was supplemented by KH_2PO_4
17.9. ¼ BBM that was supplemented by Na_2SO_4

Table 28. Experiment 17 cell density.

Day	17.1. Quarter concentration of BBM (1/4 BBM)					17.2. ¼ BBM that was supplemented by $NaNO_3$				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	276250	208750	242500	47729	20%	203750	252500	228125	34471	15%
1	347500	341250	344375	4419	1%	386250	343750	365000	30052	8%
2	837500	800000	818750	26516	3%	825000	942500	883750	83085	9%
3	1675000	1575000	1625000	70710	4%	1725000	1645000	1685000	56569	3%
4	3262500	3212500	3237500	35355	1%	2946429	2985714	2966071	27779	1%
5	4675000	5075000	4875000	282842	6%	3850000	4295238	4072619	314831	8%
6	5675000	4875000	5275000	565685	11%	6023810	5369048	5696429	462987	8%
7	5575000	5850000	5712500	194454	3%	9088095	8747619	8917857	240753	3%
8	7975000	7000000	7487500	689429	9%	12204762	12388095	12296429	129636	1%
9	7275000	7200000	7237500	53033	1%	17495238	16735714	17115476	537064	3%
10	7925000	7375000	7650000	388908	5%	21476190	20166667	20821429	925973	4%
11	7450000	7675000	7562500	159099	2%	22995238	20900000	21947619	1481557	7%
12	8000000	7750000	7875000	176776	2%	25980952	22995238	24488095	2111219	9%

Day	17.3. ¼ BBM that was supplemented by KCl					17.4. ¼ BBM that was supplemented by Fe-Na-EDTA				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	238750	228750	233750	7071	3%	267500	200000	233750	47730	20%
1	398750	373750	386250	17678	5%	346250	416250	381250	49497	13%
2	1057500	1055000	1056250	1768	0%	796250	945000	870625	105182	12%
3	1965000	1735000	1850000	162635	9%	1592500	1890000	1741250	210364	12%
4	3391667	2422619	2907143	685220	24%	3142857	3025000	3083929	83338	3%
5	3771429	3876190	3823810	74078	2%	3588095	3326190	3457143	185195	5%
6	4583333	5159524	4871429	407428	8%	5395238	5028571	5211905	259272	5%
7	5264286	5342857	5303571	55558	1%	5578571	4635714	5107143	666701	13%
8	5002381	5107143	5054762	74078	1%	7883333	7726190	7804762	111117	1%
9	6940476	7857143	7398810	648181	9%	7045238	7385714	7215476	240753	3%
10	5211905	6180952	5696429	685220	12%	7307143	7150000	7228571	111117	2%
11	5788095	6207143	5997619	296311	5%	8197619	7045238	7621429	814856	11%
12	6154762	5945238	6050000	148156	2%	8066667	6364286	7215476	1203765	17%
Day	17.5. ¼ BBM that was supplemented by $MgSO_4$					17.6. ¼ BBM that was supplemented by H_3BO_3				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	266250	165000	215625	71595	33%	190000	197500	193750	5303	3%
1	436250	453750	445000	12374	3%	420000	468750	444375	34471	8%
2	958750	953750	956250	3536	0%	823750	872500	848125	34471	4%
3	1917500	1907500	1912500	7071	0%	1647500	1745000	1696250	68943	4%
4	3142857	2841667	2992262	212974	7%	2645238	2763095	2704167	83338	3%
5	3614286	3535714	3575000	55558	2%	2880952	2802381	2841667	55558	2%
6	5238095	4819048	5028571	296311	6%	2592857	2959524	2776190	259272	9%
7	5447619	5264286	5355952	129636	2%	2435714	3326190	2880952	629662	22%
8	6050000	5683333	5866667	259272	4%	3247619	3928571	3588095	481506	13%
9	7438095	6128571	6783333	925973	14%	4373810	3954762	4164286	296311	7%
10	7385714	7642381	7514048	181491	2%	4216667	3986190	4101429	162971	4%
11	7202381	7841429	7521905	451875	6%	3823810	4950000	4386905	796337	18%
12	6757143	7464286	7110714	500026	7%	3850000	5054762	4452381	851895	19%

Day	17.7. ¼ BBM that was supplemented by $CaCl_2$					17.8. ¼ BBM that was supplemented by KH_2PO_4				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	207500	201250	204375	4419	2%	251250	251250	251250	0	0%
1	342500	471250	406875	91040	22%	427500	423750	425625	2652	1%
2	960000	1057500	1008750	68943	7%	1035000	1015000	1025000	14142	1%
3	1895000	2115000	2005000	155563	8%	1907500	2030000	1968750	86621	4%
4	3064286	4020238	3542262	675960	19%	3142857	3195238	3169048	37039	1%
5	4950000	3902381	4426190	740779	17%	3588095	3771429	3679762	129636	4%
6	4609524	5054762	4832143	314831	7%	4373810	4557143	4465476	129636	3%
7	4766667	5971429	5369048	851895	16%	4976190	4400000	4688095	407428	9%
8	6259524	7019048	6639286	537064	8%	6704762	6154762	6429762	388909	6%
9	5185714	6154762	5670238	685220	12%	6338095	8642857	7490476	1629713	22%
10	5761905	7590000	6675952	1292659	19%	7804762	7307143	7555952	351870	5%
11	5211905	6547619	5879762	944493	16%	8511905	6730952	7621429	1259324	17%
12	5080952	7647619	6364286	1814907	29%	8590476	6652381	7621429	1370440	18%

Day	17.9. ¼ BBM that was supplemented by Na_2SO_4				
	Q	R	Average	SD	SE
0	220000	218750	219375	884	0%
1	415000	476250	445625	43310	10%
2	965000	953750	959375	7955	1%
3	1930000	1907500	1918750	15910	1%
4	3836905	3313095	3575000	370389	10%
5	3980952	4740476	4360714	537064	12%
6	5919048	6076190	5997619	111117	2%
7	5054762	4897619	4976190	111117	2%
8	5735714	6259524	5997619	370389	6%
9	6050000	6547619	6298810	351870	6%
10	6311905	6678571	6495238	259272	4%
11	7045238	6888095	6966667	111117	2%
12	7804762	7019048	7411905	555584	7%

Table 29. Experiment 17 pH change

Culture	17.1. 1/4 BBM		17.2. $NaNO_3$		17.3. KCl		17.4. Na Fe EDTA		17.5. $MgSO_4$		17.6. H_3BO_3		17.7. $CaCl_2$		17.8. KH_2PO_4		17.9. Na_2SO_4	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Initial pH	6.58		6.58		6.58		6.58		6.58		6.58		6.58		6.58		6.58	
Final pH	6.69	6.71	7.16	7.22	6.29	6.68	6.6	6.52	6.54	6.54	6.43	6.45	6.29	6.43	5.89	5.85	6.46	6.36
Average	6.7		7.19		6.485		6.56		6.54		6.44		6.36		5.87		6.41	
SD	0.0141		0.0424		0.2758		0.0566		0.0000		0.0141		0.0990		0.0283		0.0707	
SE	0%		1%		4%		1%		0%		0%		2%		0%		1%	

Table 30. Experiment 17 biomass dry weight

Culture	17.1. 1/4 BBM		17.2. $NaNO_3$		17.3. KCl		17.4. Na Fe EDTA		17.5. $MgSO_4$		17.6. H_3BO_3		17.7. $CaCl_2$		17.8. KH_2PO_4		17.9. Na_2SO_4	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Mass (g)	1.068	0.96	2.368	2.46	0.676	0.908	1.156	1.184	1.072	1.092	0.344	0.472	1.008	1.164	0.996	1.02	1.08	1.128
Average	1.014		2.414		0.792		1.17		1.082		0.408		1.086		1.008		1.104	
SD	0.076		0.0651		0.1640		0.0198		0.0141		0.0905		0.1103		0.0170		0.034	
SE	8%		3%		21%		2%		1%		22%		10%		2%		3%	

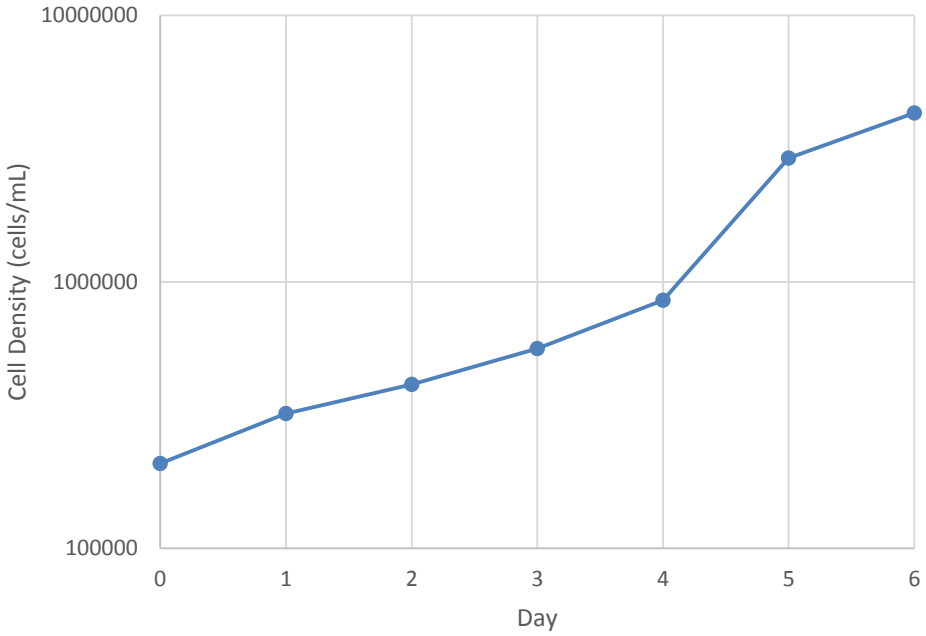

Figure 106. Growth curve of T. sp. LCR-Awa9/2 inoculum to start experiment 17.

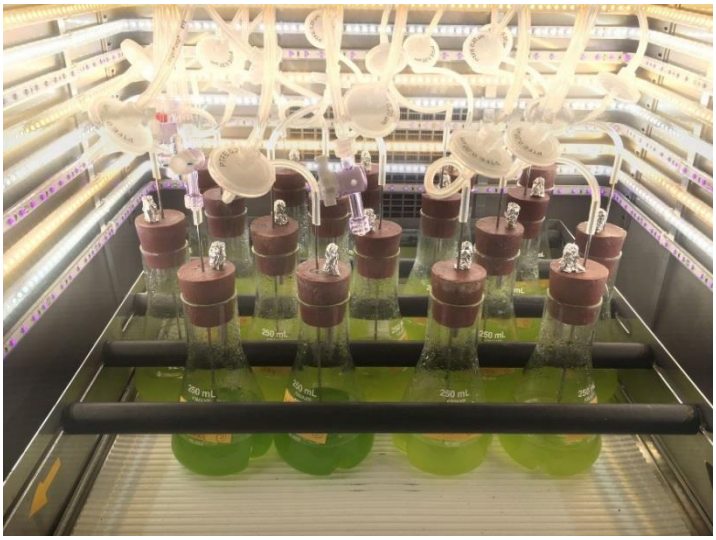

Figure 107. Experiment 17

Figure 108. Environmental algae in experiment 17.

Experiment 19

19.1. ¼BBM4N
19.2. ¼BBM4N without micronutrients
19.3. ¼BBM4N without boron
19.4. ¼BBM4N without iron and EDTA
19.5. ¼BBM4N without zinc
19.6. ¼BBM4N without manganese
19.7. ¼BBM4N without molybdate
19.8. ¼BBM4N without copper
19.9. ¼BBM4N without cobalt

Table 31. Experiment 19 cell density.

Day	19.1. ¼BBM4N					19.2. ¼BBM4N without micronutrients				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	265000	273750	269375	6187	2%	272500	270000	271250	1768	1%
1	527500	417500	472500	77782	16%	395000	445000	420000	35355	8%
2	892500	746250	819375	103414	13%	810000	762500	786250	33588	4%
3	1503750	1687500	1595625	129931	8%	1533750	1353750	1443750	127279	9%
4	2562500	3387500	2975000	583363	20%	2800000	2825000	2812500	17678	1%
5	4937500	5500000	5218750	397748	8%	5712500	5375000	5543750	238649	4%
6	9050000	9212500	9131250	114905	1%	5875000	7162500	6518750	910400	14%
7	13125000	11850000	12487500	901561	7%	5525000	7600000	6562500	1467247	22%
8	15100000	16850000	15975000	1237437	8%	8800000	9000000	8900000	141421	2%
9	22500000	20800000	21650000	1202082	6%	11400000	8500000	9950000	2050610	21%
10	28350000	25725000	27037500	1856155	7%	9150000	9525000	9337500	265165	3%
11	25950000	28275000	27112500	1644023	6%	6675000	7350000	7012500	477297	7%
12	29400000	29850000	29625000	318198	1%	9600000	10050000	9825000	318198	3%
13	31950000	32475000	32212500	371231	1%	11700000	13200000	12450000	1060660	9%
14	30000000	31200000	30600000	848528	3%	10425000	12375000	11400000	1378858	12%

Day	19.3. ¼BBM4N without boron					19.4. ¼BBM4N without iron and EDTA				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	255000	265000	260000	7071	3%	275000	258750	266875	11490	4%
1	480000	477500	478750	1768	0%	440000	437500	438750	1768	0%
2	948750	941250	945000	5303	1%	787500	742500	765000	31820	4%
3	1852500	1680000	1766250	121976	7%	1511250	1642500	1576875	92808	6%
4	2900000	2625000	2762500	194454	7%	3550000	3000000	3275000	388909	12%
5	5612500	5850000	5731250	167938	3%	5612500	6000000	5806250	274004	5%
6	8687500	8050000	8368750	450781	5%	9900000	9875000	9887500	17678	0%
7	13675000	12925000	13300000	530330	4%	15200000	14800000	15000000	282843	2%
8	15300000	18900000	17100000	2545584	15%	19000000	20900000	19950000	1343503	7%
9	21200000	22700000	21950000	1060660	5%	22100000	23000000	22550000	636396	3%
10	24825000	22425000	23625000	1697056	7%	28270000	27525000	27897500	526795	2%
11	28950000	25125000	27037500	2704683	10%	26850000	28350000	27600000	1060660	4%
12	28800000	27600000	28200000	848528	3%	28200000	28650000	28425000	318198	1%
13	26550000	29475000	28012500	2068287	7%	30075000	29250000	29662500	583363	2%
14	32400000	32325000	32362500	53033	0%	30300000	29175000	29737500	795495	3%
Day	19.5. ¼BBM4N without zinc					19.6. ¼BBM4N without manganese				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	262500	236250	249375	18562	7%	293750	250000	271875	30936	11%
1	452500	465000	458750	8839	2%	447500	440000	443750	5303	1%
2	828750	855000	841875	18562	2%	798750	716250	757500	58336	8%
3	1702500	1635000	1668750	47730	3%	1653750	1605000	1629375	34471	2%
4	2837500	3425000	3131250	415425	13%	3225000	3925000	3575000	494975	14%
5	5300000	4837500	5068750	327037	6%	5462500	5437500	5450000	17678	0%
6	8925000	8375000	8650000	388909	4%	8237500	7025000	7631250	857367	11%
7	14425000	13050000	13737500	972272	7%	9875000	8675000	9275000	848528	9%
8	18100000	19550000	18825000	1025305	5%	12400000	9500000	10950000	2050610	19%
9	19800000	19300000	19550000	353553	2%	13050000	12850000	12950000	141421	1%
10	22950000	33975000	28462500	7795852	27%	14850000	13800000	14325000	742462	5%
11	23025000	24000000	23512500	689429	3%	16200000	15600000	15900000	424264	3%
12	24000000	25800000	24900000	1272792	5%	16350000	16050000	16200000	212132	1%
13	24825000	26375000	25600000	1096016	4%	16650000	19275000	17962500	1856155	10%
14	26625000	24650000	25637500	1396536	5%	18225000	19125000	18675000	636396	3%

Day	19.7. ¼BBM4N without molybdate					19.8. ¼BBM4N without copper				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	255000	266250	260625	7955	3%	301250	291250	296250	7071	2%
1	447500	512500	480000	45962	10%	427500	462500	445000	24749	6%
2	813750	877500	845625	45078	5%	795000	723750	759375	50381	7%
3	1946250	1676250	1811250	190919	11%	1743750	1773750	1758750	21213	1%
4	2925000	2850000	2887500	53033	2%	3275000	2950000	3112500	229810	7%
5	5750000	4825000	5287500	654074	12%	5112500	4512500	4812500	424264	9%
6	8812500	8700000	8756250	79550	1%	7800000	7737500	7768750	44194	1%
7	15250000	15875000	15562500	441942	3%	11700000	12575000	12137500	618718	5%
8	17400000	18100000	17750000	494975	3%	18000000	13950000	15975000	2863782	18%
9	20300000	19400000	19850000	636396	3%	21500000	19500000	20500000	1414214	7%
10	24075000	29400000	26737500	3765344	14%	28050000	24750000	26400000	2333452	9%
11	25275000	25800000	25537500	371231	1%	23250000	24000000	23625000	530330	2%
12	27600000	28950000	28275000	954594	3%	28050000	26400000	27225000	1166726	4%
13	32400000	31950000	32175000	318198	1%	32475000	26700000	29587500	4083542	14%
14	33150000	31500000	32325000	1166726	4%	34950000	30975000	32962500	2810749	9%

Day	19.9. ¼BBM4N without cobalt				
	Q	R	Average	SD	SE
0	251250	283750	267500	22981	9%
1	410000	367500	388750	30052	8%
2	787500	877500	832500	63640	8%
3	1702500	1773750	1738125	50381	3%
4	2937500	3125000	3031250	132583	4%
5	5350000	5762500	5556250	291682	5%
6	7637500	7800000	7718750	114905	1%
7	11250000	12775000	12012500	1078338	9%
8	16550000	15450000	16000000	777817	5%
9	21900000	18450000	20175000	2439518	12%
10	23025000	20400000	21712500	1856155	9%
11	24225000	25200000	24712500	689429	3%
12	26230000	28800000	27515000	1817264	7%
13	29850000	22647500	26248750	5092937	19%
14	31050000	32325000	31687500	901561	3%

Table 32. Experiment 19 pH change

Culture	19.1. Control		19.2. D-Trace Element		19.3. H_3BO_3 Depleted		19.4. EDTA Depleted		19.5. $ZnSO_4$ Depleted		19.6. $MnCl_2$ Depleted		19.7. MoO_3 Depleted		19.8. $CuSO_4$ Depleted		19.9. $CO(NO_3)_2$-D	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Initial pH	6.35		6.39		6.33		6.55		6.45		6.39		6.54		6.57		6.39	
Final pH	7.37	7.24	7.21	7.01	6.87	7.14	7.17	7.19	6.96	7.14	7.13	7.09	7.09	7.08	7.015	7.15	6.95	6.99
Average	7.305		7.11		7.005		7.18		7.05		7.11		7.085		7.0825		6.97	
SD	0.0919		0.1414		0.1909		0.0141		0.1273		0.0283		0.0071		0.0955		0.0283	
SE	1%		2%		3%		0%		2%		0%		0%		1%		0%	

Table 33. Experiment 19 biomass dry weight

Culture	19.1. Control		19.2. D-Trace Element		19.3. H_3BO_3 Depleted		19.4. EDTA Depleted		19.5. $ZnSO_4$ Depleted		19.6. $MnCl_2$ Depleted		19.7. MoO_3 Depleted		19.8. $CuSO_4$ Depleted		19.9. $CO(NO_3)_2$-D	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Mass (g)	2.275	2.257	0.715	0.755	1.976	1.99	1.883	2.025	1.969	2.199	1.181	0.781	2.534	2.617	2.719	2.387	2.288	2.212
Average	2.266		0.735		1.983		1.954		2.084		0.981		2.5755		2.553		2.25	
SD	0.0127		0.0283		0.0099		0.1004		0.1626		0.2828		0.0587		0.2348		0.0537	
SE	1%		4%		0%		5%		8%		29%		2%		9%		2%	

Figure 109. Environmental alga in ¼ Bold Basal Medium which was supplemented with 4× $NaNO_3$ concentration, with eight different culture media conditions (exp.19)

Experiment 20

20.1. BBM2N
20.2. BBM2N N source: Urea
20.3. BBM2N N source: Urea+$NiCl_2$ (Ni concentration 5×10^{-7} M)
20.4. BBM2N N source: Ammonium chloride
20.5. BBM2N that used $FeCl_3$ and NaCitrate as iron source and chelating agent
20.6. BBM2N that used $FeSO_4$ and Na_2EDTA as iron source and chelating agent
20.7. BBM2N that used $FeCl_3$ as iron source
20.8. BBM2N1/32P
20.9. BBM2N that was supplemented by vitamins
20.10. BBM2N that was supplemented by selenium dioxide
20.11. BBM2N that was supplemented by selenous acid

Table 34. Experiment 20 cell density.

Day	20.1. BBM2N					20.2. BBM2N N source: Urea				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	265000	181250	223125	59220	27%	202500	225000	213750	15910	7%
1	270000	328750	299375	41543	14%	273750	321250	297500	33588	11%
2	741250	715000	728125	18562	3%	518750	543750	531250	17678	3%
3	1172500	1252500	1212500	56569	5%	712500	710000	711250	1768	0%
4	2562500	2612500	2587500	35355	1%	1112500	950000	1031250	114905	11%
5	4250000	3512500	3881250	521491	13%	1562500	1475000	1518750	61872	4%
6	7075000	5450000	6262500	1149049	18%	5725000	5000000	5362500	512652	10%
7	8500000	9750000	9125000	883883	10%	5925000	6600000	6262500	477297	8%
8	14920000	13031250	13975625	1335548	10%	8975000	10056250	9515625	764559	8%
9	16375000	15000000	15687500	972272	6%	13050000	13300000	13175000	176777	1%
10	18600000	17800000	18200000	565685	3%	15500000	15900000	15700000	282843	2%
11	20480000	20800000	20640000	226274	1%	17190000	18500000	17845000	926310	5%
12	24050000	24050000	24050000	0	0%	20650000	23500000	22075000	2015254	9%
13	27900000	27000000	27450000	636396	2%	25200000	27450000	26325000	1590990	6%
14	30600000	31500000	31050000	636396	2%	31800000	32850000	32325000	742462	2%
15	34500000	35100000	34800000	424264	1%	38250000	39600000	38925000	954594	2%
16	36375000	35850000	36112500	371231	1%	39600000	43200000	41400000	2545584	6%

Day	20.3. BBM2N N source: Urea+$NiCl_2$					20.4. BBM2N N source: Ammonium chloride				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	233750	207500	220625	18562	8%	283750	225000	254375	41543	16%
1	251250	236250	243750	10607	4%	335000	338750	336875	2652	1%
2	516250	537500	526875	15026	3%	662500	676250	669375	9723	1%
3	785000	812500	798750	19445	2%	967500	992500	980000	17678	2%
4	1750000	1637500	1693750	79550	5%	2625000	2525000	2575000	70711	3%
5	2087500	3262500	2675000	830850	31%	4637500	3700000	4168750	662913	16%
6	3825000	5325000	4575000	1060660	23%	8350000	6825000	7587500	1078338	14%
7	9100000	9550000	9325000	318198	3%	10125000	11275000	10700000	813173	8%
8	11750000	12050000	11900000	212132	2%	13250000	13900000	13575000	459619	3%
9	12350000	16300000	14325000	2793072	19%	17500000	14400000	15950000	2192031	14%
10	18100000	19500000	18800000	989949	5%	17500000	18100000	17800000	424264	2%
11	23000000	24500000	23750000	1060660	4%	17900000	19300000	18600000	989949	5%
12	28400000	30050000	29225000	1166726	4%	18800000	21650000	20225000	2015254	10%
13	31125000	32250000	31687500	795495	3%	18750000	18450000	18600000	212132	1%
14	36150000	38700000	37425000	1803122	5%	20550000	19650000	20100000	636396	3%
15	39300000	41250000	40275000	1378858	3%	21600000	22050000	21825000	318198	1%
16	39600000	46100000	42850000	4596194	11%	23625000	23100000	23362500	371231	2%
Day	20.5. BBM2N FeCl3 + Na-Citrate					20.6. BBM2N $FeSO_4$ + Na_2EDTA				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	232500	210000	221250	15910	7%	231250	225000	228125	4419	2%
1	318750	370000	344375	36239	11%	303750	326250	315000	15910	5%
2	667500	682500	675000	10607	2%	653750	637500	645625	11490	2%
3	1030000	1045000	1037500	10607	1%	1142500	832500	987500	219203	22%
4	2275000	2400000	2337500	88388	4%	2450000	2250000	2350000	141421	6%
5	3637500	3450000	3543750	132583	4%	3575000	4237500	3906250	468458	12%
6	6125000	8275000	7200000	1520280	21%	7325000	6500000	6912500	583363	8%
7	10800000	9775000	10287500	724784	7%	10925000	11450000	11187500	371231	3%
8	17050000	15606250	16328125	1020885	6%	17750000	16631250	17190625	791076	5%
9	23200000	20700000	21950000	1767767	8%	23350000	19750000	21550000	2545584	12%
10	24000000	21300000	22650000	1909188	8%	26600000	23600000	25100000	2121320	8%
11	27700000	32000000	29850000	3040559	10%	28420000	27000000	27710000	1004092	4%
12	31400000	35950000	33675000	3217336	10%	31150000	29150000	30150000	1414214	5%
13	38550000	42525000	40537500	2810749	7%	34425000	35250000	34837500	583363	2%
14	39750000	44250000	42000000	3181981	8%	35250000	35550000	35400000	212132	1%
15	41850000	45450000	43650000	2545584	6%	35850000	37050000	36450000	848528	2%
16	44920000	49875000	47397500	3503714	7%	37275000	37800000	37537500	371231	1%

Day	20.7. BBM2N that used $FeCl_3$ as iron source					20.8. BBM2N1/32P				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	237500	207500	222500	21213	10%	210000	225000	217500	10607	5%
1	335000	381250	358125	32704	9%	370000	347500	358750	15910	4%
2	1037500	900000	968750	97227	10%	681250	872500	776875	135234	17%
3	1197500	997500	1097500	141421	13%	942500	1207500	1075000	187383	17%
4	2525000	2862500	2693750	238649	9%	2812500	3062500	2937500	176777	6%
5	3887500	4162500	4025000	194454	5%	4387500	5225000	4806250	592202	12%
6	7100000	7475000	7287500	265165	4%	6675000	7000000	6837500	229810	3%
7	12000000	9450000	10725000	1803122	17%	9300000	9825000	9562500	371231	4%
8	13275000	12337500	12806250	662913	5%	13350000	12956250	13153125	278423	2%
9	16700000	13300000	15000000	2404163	16%	16000000	15650000	15825000	247487	2%
10	23800000	23000000	23400000	565685	2%	19400000	18000000	18700000	989949	5%
11	23400000	22000000	22700000	989949	4%	21100000	19700000	20400000	989949	5%
12	28150000	25550000	26850000	1838478	7%	23050000	20600000	21825000	1732412	8%
13	35025000	32550000	33787500	1750089	5%	24000000	21000000	22500000	2121320	9%
14	37200000	34950000	36075000	1590990	4%	24600000	22350000	23475000	1590990	7%
15	41250000	37350000	39300000	2757716	7%	25800000	23850000	24825000	1378858	6%
16	46725000	44325000	45525000	1697056	4%	29400000	27150000	28275000	1590990	6%
Day	20.9. BBM2N that was supplemented by vitamins					20.10. BBM2N that was supplemented by selenium dioxide				
	Q	R	Average	SD	SE	S	T	Average	SD	SE
0	200000	221250	210625	15026	7%	211250	178750	195000	22981	12%
1	235000	308750	271875	52149	19%	311250	366250	338750	38891	11%
2	637500	627500	632500	7071	1%	787500	843750	815625	39775	5%
3	947500	1117500	1032500	120208	12%	1167500	1297500	1232500	91924	7%
4	2625000	3062500	2843750	309359	11%	2900000	3250000	3075000	247487	8%
5	4525000	3612500	4068750	645235	16%	3962500	4412500	4187500	318198	8%
6	7275000	8050000	7662500	548008	7%	5850000	5985000	5917500	95459	2%
7	11300000	11075000	11187500	159099	1%	9775000	10100000	9937500	229810	2%
8	14950000	13731250	14340625	861786	6%	11050000	12606250	11828125	1100435	9%
9	17800000	15200000	16500000	1838478	11%	16100000	13800000	14950000	1626346	11%
10	21200000	20000000	20600000	848528	4%	19800000	17000000	18400000	1979899	11%
11	27400000	24700000	26050000	1909188	7%	21700000	19600000	20650000	1484924	7%
12	33200000	29550000	31375000	2580940	8%	25950000	28900000	27425000	2085965	8%
13	34500000	35250000	34875000	530330	2%	32475000	34950000	33712500	1750089	5%
14	34800000	37050000	35925000	1590990	4%	32250000	35250000	33750000	2121320	6%
15	37800000	38250000	38025000	318198	1%	33000000	36000000	34500000	2121320	6%
16	39375000	40800000	40087500	1007627	3%	34425000	38775000	36600000	3075914	8%

Day	20.11. BBM2N that was supplemented by selenous acid				
	U	V	Average	SD	SE
0	212500	195000	203750	12374	6%
1	310000	371250	340625	43310	13%
2	593750	575000	584375	13258	2%
3	797500	905000	851250	76014	9%
4	1912500	2112500	2012500	141421	7%
5	3275000	2537500	2906250	521491	18%
6	4200000	3975000	4087500	159099	4%
7	6050000	6600000	6325000	388909	6%
8	9725000	8512500	9118750	857367	9%
9	11350000	8950000	10150000	1697056	17%
10	13600000	11200000	12400000	1697056	14%
11	17000000	14800000	15900000	1555635	10%
12	25250000	22850000	24050000	1697056	7%
13	26175000	27750000	26962500	1113693	4%
14	27600000	29550000	28575000	1378858	5%
15	28800000	31650000	30225000	2015254	7%
16	28275000	35850000	32062500	5356334	17%

Table 35. Experiment 20 pH change

Culture	20.1. Control: BBM2N		20.2. BBM2N (N source: Urea)		20.3. BBM2N (N source: Urea + Nickel)		20.4. BBM2N (N source: Ammonia)		20.5. (Fe source: FeCl3 + NaCitrate)		20.6. (Fe source: FeSO4 + Na2EDTA)	
	A	B	C	D	E	F	G	H	I	J	K	L
Final pH	7.95	7.15	6.71	7.35	6.44	6.17	3.09	3.18	7.2	7.12	7.06	7.18
Average	7.55		7.03		6.305		3.135		7.16		7.12	
SD	0.5657		0.4525		0.1909		0.0636		0.0566		0.0849	
SE	7%		6%		3%		2%		1%		1%	
Culture	20.7. (Fe source: FeCl3)		20.8. BBM2N 1/32 Phosphate		20.9. BBM2N (OHM'S Vitamins)		20.10. BBM2N (SeO2)		20.11. BBM2N (H2SeO3)			
	M	N	O	P	Q	R	S	T	U	V		
Final pH	7.08	7.03	7.03	6.91	7.07	7.09	7.07	7.07	7.07	7.06		
Average	7.055		6.97		7.08		7.07		7.065			
SD	0.0354		0.0849		0.0141		0.0000		0.0071			
SE	1%		1%		0%		0%		0%			

Table 36. Experiment 20 biomass dry weight

Culture	20.1. Control: BBM2N		20.2. BBM2N (N source: Urea)		20.3. BBM2N (N source: Urea + Nickel)		20.4. BBM2N (N source: Ammonia)		20.5. (Fe source: FeCl3 + NaCitrate)		20.6. (Fe source: FeSO4 + Na2EDTA)	
	A	B	C	D	E	F	G	H	I	J	K	L
Mass (g)	2.565347	2.258416	2.3821782	2.50495	2.40297	2.738614	1.615842	1.859406	2.513861	2.521782	2.917822	2.937624
Average	2.411		2.443		2.570		1.737		2.517		2.927	
SD	0.2170		0.0868		0.2373		0.1722		0.0056		0.0140	
SE	9%		4%		9%		10%		0%		0%	
Culture	20.7. (Fe source: FeCl3)		20.8. BBM2N 1/32 Phosphate		20.9. BBM2N (OHM'S Vitamins)		20.10. BBM2N (SeO2)		20.11. BBM2N (H2SeO3)			
	M	N	O	P	Q	R	S	T	U	V		
Mass (g)	2.555	2.380198	2.746	2.556436	2.424752	2.174257	2.563	2.755	1.902	2.325		
Average	2.467		2.651		2.299		2.659		2.1135			
SD	0.1236		0.1340		0.1771		0.1358		0.2991			
SE	5%		5%		8%		5%		14%			

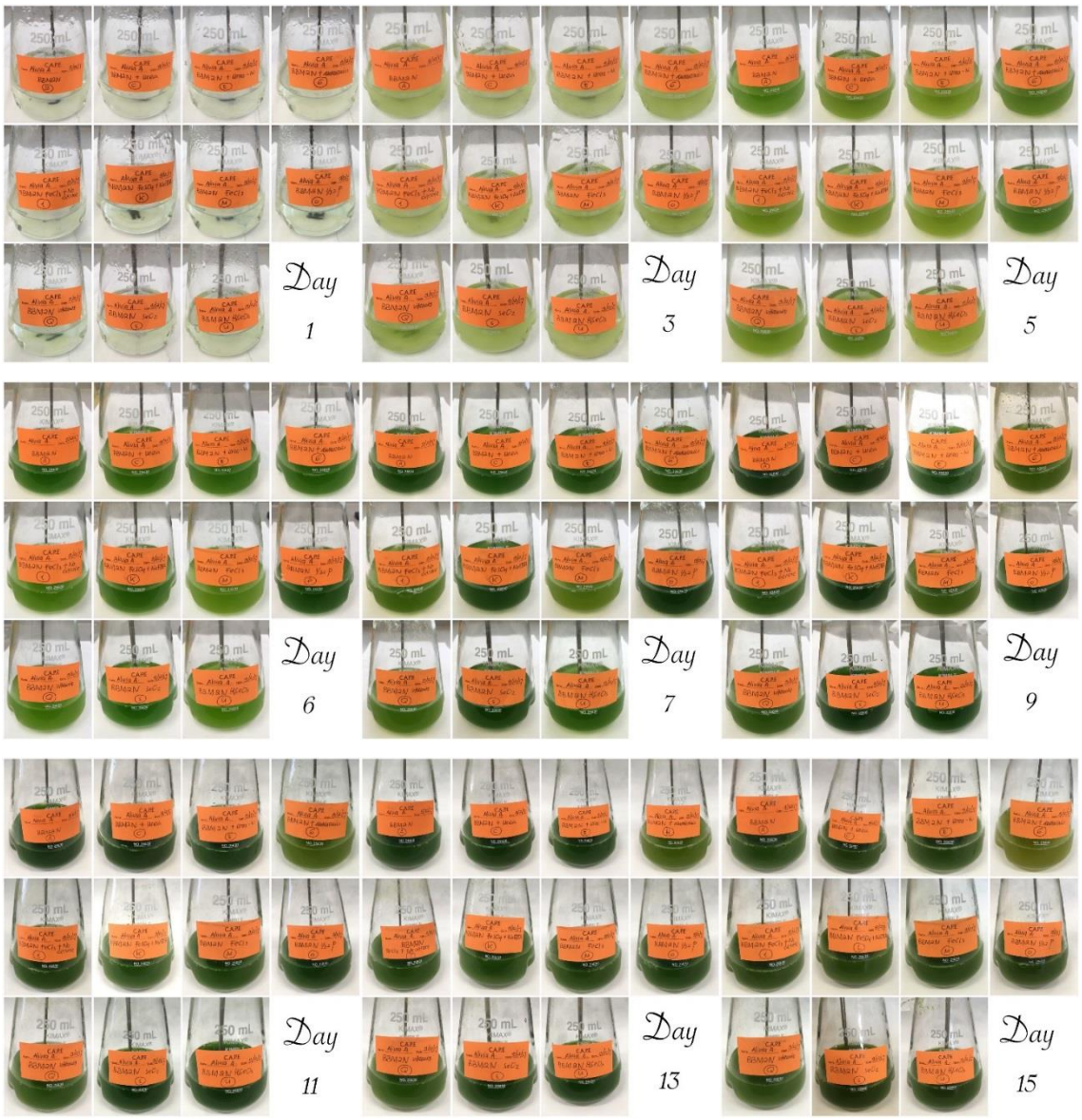

Figure 110. Experiment 20 cultures.

Experiment 21

Experimental conditions:
21.1. Control medium,
21.2. Culture that shrouded by white plastic resulting in 2/3 light entered the flask,
21.3. Culture that shrouded by white plastic until day 5, resulting in 1/2 light entered the flask,
21.4., 21.5, 21.6. Culture that shrouded by cellophane green, red, and blue,
21.7., 21.8., 21.9. Culture that shrouded by cellophane green, red, and blue after cell density reached 3×107 cells/mL,
21.10. Culture that shrouded by white plastic resulting in medium light intensity after cell density reached 3×107 cells/mL.

Table 37. Experiment 21 cell density.

Day	21.1. Control medium					21.2. 2/3 light				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	238750	210000	224375	20329	9%	213750	210000	211875	2652	1%
1	373750	461250	417500	61872	15%	422500	416250	419375	4419	1%
2	937500	887500	912500	35355	4%	907500	770000	838750	97227	12%
3	1925000	2230000	2077500	215668	10%	2030000	2150000	2090000	84853	4%
4	3785000	3975000	3880000	134350	3%	4450000	4175000	4312500	194454	5%
5	6925000	7025000	6975000	70711	1%	6825000	6275000	6550000	388909	6%
6	10850000	11250000	11050000	282843	3%	8800000	8400000	8600000	282843	3%
7	15300000	15900000	15600000	424264	3%	11200000	9725000	10462500	1042983	10%
8	21300000	16600000	18950000	3323402	18%	20050000	19300000	19675000	530330	3%
9	28300000	21700000	25000000	4666905	19%	31450000	31600000	31525000	106066	0%
10	29900000	29200000	29550000	494975	2%	36000000	36400000	36200000	282843	1%
11	35000000	33700000	34350000	919239	3%	40000000	41600000	40800000	1131371	3%
12	38700000	41925000	40312500	2280419	6%	42450000	48150000	45300000	4030509	9%
13	41600000	43600000	42600000	1414214	3%	49600000	50400000	50000000	565685	1%
14	42400000	44400000	43400000	1414214	3%	54200000	55800000	55000000	1131371	2%
15	42700000	45100000	43900000	1697056	4%	57200000	58600000	57900000	989949	2%
16	43650000	46575000	45112500	2068287	5%	57225000	59925000	58575000	1909188	3%

Day	21.3. ½ light					21.4. Green				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	271250	231250	251250	28284	11%	226250	267500	246875	29168	12%
1	445000	417500	431250	19445	5%	442500	410000	426250	22981	5%
2	802500	802500	802500	0	0%	690000	717500	703750	19445	3%
3	1750000	1940000	1845000	134350	7%	2055000	2070000	2062500	10607	1%
4	3350000	3825000	3587500	335876	9%	4637500	4250000	4443750	274004	6%
5	5875000	5425000	5650000	318198	6%	6000000	5925000	5962500	53033	1%
6	6750000	6750000	6750000	0	0%	10150000	8200000	9175000	1378858	15%
7	8500000	8225000	8362500	194454	2%	10125000	12050000	11087500	1361181	12%
8	17500000	16950000	17225000	388909	2%	13900000	16950000	15425000	2156676	14%
9	23200000	25600000	24400000	1697056	7%	16150000	18950000	17550000	1979899	11%
10	29600000	29300000	29450000	212132	1%	22500000	21200000	21850000	919239	4%
11	33600000	32400000	33000000	848528	3%	26000000	25700000	25850000	212132	1%
12	39825000	33450000	36637500	4507806	12%	31800000	28200000	30000000	2545584	8%
13	43800000	45000000	44400000	848528	2%	35200000	31200000	33200000	2828427	9%
14	50900000	49200000	50050000	1202082	2%	39800000	34700000	37250000	3606245	10%
15	53200000	53600000	53400000	282843	1%	42600000	37250000	39925000	3783021	9%
16	54450000	56625000	55537500	1537957	3%	44025000	39825000	41925000	2969848	7%
Day	21.5. Red					21.6. Blue				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	283750	207500	245625	53917	22%	232500	242500	237500	7071	3%
1	415000	456250	435625	29168	7%	385000	403750	394375	13258	3%
2	935000	885000	910000	35355	4%	787500	870000	828750	58336	7%
3	1845000	2160000	2002500	222739	11%	2110000	1755000	1932500	251023	13%
4	2900000	3837500	3368750	662913	20%	4525000	3962500	4243750	397748	9%
5	4937500	6125000	5531250	839689	15%	6675000	7275000	6975000	424264	6%
6	5750000	7250000	6500000	1060660	16%	9350000	10350000	9850000	707107	7%
7	6600000	8925000	7762500	1644023	21%	12750000	13675000	13212500	654074	5%
8	9950000	11000000	10475000	742462	7%	16200000	19150000	17675000	2085965	12%
9	13850000	12950000	13400000	636396	5%	28350000	26850000	27600000	1060660	4%
10	14350000	14600000	14475000	176777	1%	32000000	33300000	32650000	919239	3%
11	15400000	16750000	16075000	954594	6%	36950000	38000000	37475000	742462	2%
12	14100000	19575000	16837500	3871410	23%	39300000	42225000	40762500	2068287	5%
13	18250000	20400000	19325000	1520280	8%	42200000	43900000	43050000	1202082	3%
14	19750000	24000000	21875000	3005204	14%	44080000	45250000	44665000	827315	2%
15	21725000	26600000	24162500	3447146	14%	45750000	42050000	43900000	2616295	6%
16	22725000	28975000	25850000	4419417	17%	46650000	39675000	43162500	4932070	11%

Day	21.7. Green after 1e7					21.8. Red after 1e7				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	227500	221250	224375	4419	2%	223750	236250	230000	8839	4%
1	415000	373750	394375	29168	7%	370000	396250	383125	18562	5%
2	762500	820000	791250	40659	5%	675000	795000	735000	84853	12%
3	1945000	1980000	1962500	24749	1%	2165000	1875000	2020000	205061	10%
4	4162500	4020000	4091250	100763	2%	3980000	4637500	4308750	464923	11%
5	8512500	7975000	8243750	380070	5%	7162500	8237500	7700000	760140	10%
6	10300000	11500000	10900000	848528	8%	9600000	10350000	9975000	530330	5%
7	20900000	19000000	19950000	1343503	7%	16850000	17250000	17050000	282843	2%
8	29650000	26390000	28020000	2305168	8%	26250000	25200000	25725000	742462	3%
9	41550000	35000000	38275000	4631549	12%	33050000	33850000	33450000	565685	2%
10	40525000	39200000	39862500	936916	2%	36400000	37250000	36825000	601041	2%
11	40400000	41250000	40825000	601041	1%	39600000	40400000	40000000	565685	1%
12	41100000	42750000	41925000	1166726	3%	42375000	43050000	42712500	477297	1%
13	42240000	49800000	46020000	5345727	12%	45200000	46250000	45725000	742462	2%
14	44880000	45250000	45065000	261630	1%	47600000	53250000	50425000	3995153	8%
15	46600000	45600000	46100000	707107	2%	49080000	47600000	48340000	1046518	2%
16	47025000	43500000	45262500	2492551	6%	50850000	46425000	48637500	3128948	6%
Day	21.9. Blue after 1e7					21.10. Dark after 1e7				
	Q	R	Average	SD	SE	S	T	Average	SD	SE
0	236250	196250	216250	28284	13%	260000	192500	226250	47730	21%
1	518750	432500	475625	60988	13%	397500	402500	400000	3536	1%
2	885000	728000	806500	111016	14%	835000	865000	850000	21213	2%
3	2230000	1890000	2060000	240416	12%	2145000	2125000	2135000	14142	1%
4	4975000	4225000	4600000	530330	12%	5225000	5400000	5312500	123744	2%
5	9080000	8240000	8660000	593970	7%	7455000	8025000	7740000	403051	5%
6	13550000	11250000	12400000	1626346	13%	9550000	11850000	10700000	1626346	15%
7	18100000	19000000	18550000	636396	3%	16250000	15300000	15775000	671751	4%
8	24500000	26800000	25650000	1626346	6%	20050000	21250000	20650000	848528	4%
9	34000000	36300000	35150000	1626346	5%	34700000	36100000	35400000	989949	3%
10	38400000	39750000	39075000	954594	2%	39200000	40400000	39800000	848528	2%
11	41600000	40400000	41000000	848528	2%	42600000	43500000	43050000	636396	1%
12	46425000	42060000	44242500	3086521	7%	45825000	47400000	46612500	1113693	2%
13	47200000	46400000	46800000	565685	1%	50800000	51600000	51200000	565685	1%
14	49200000	50250000	49725000	742462	1%	55200000	57000000	56100000	1272792	2%
15	49850000	48520000	49185000	940452	2%	57250000	59200000	58225000	1378858	2%
16	49275000	56625000	52950000	5197235	10%	59400000	69675000	64537500	7265522	11%

Table 38. Experiment 21 pH change

Culture	21.1. Control		21.2. 2/3 White		21.3. 1/2 White		21.4. Green		21.5. Blue		21.6. Red		21.7. Green CD 3X107		21.8. Blue CD 3X107		21.9. Red CD 3X107	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Initial pH	6.26		6.26		6.26		6.26		6.26		6.26		6.26		6.26		6.26	
Final pH	7.31	7.2	7.28	7.12	7.12	7.16	7.07	7.05	6.99	7.06	7.08	7.09	7.1	7.29	7.09	7.23	7.17	7.3
Average	7.255		7.2		7.14		7.06		7.025		7.085		7.195		7.16		7.235	
SD	0.0778		0.1131		0.0283		0.0141		0.0495		0.0071		0.1344		0.0990		0.0919	
SE	1%		2%		0%		0%		1%		0%		2%		1%		1%	

Culture	21.10. Dark after 3e7	
	S	T
Initial pH	6.26	
Final pH	7.11	7.14
Average	7.125	
SD	0.0212	
SE	0%	

Table 39. Experiment 21 biomass dry weight

Culture	21.1. Control		21.2. 2/3 White		21.3. 1/2 White		21.4. Green		21.5. Blue		21.6. Red		21.7. Green CD 3X107		21.8. Blue CD 3X107		21.9. Red CD 3X107	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Mass (g)	4.073	4.087	4.054	3.834	3.992	3.870	2.677	2.469	1.583	2.252	3.240	3.105	3.305	3.207	3.105	3.124	3.615	3.723
Average	4.080		3.944		3.931		2.573		1.918		3.173		3.256		3.114		3.669	
SD	0.0097		0.1553		0.0867		0.1477		0.4728		0.0957		0.0693		0.0132		0.0763	
SE	0%		4%		2%		6%		25%		3%		2%		0%		2%	

Culture	21.10. Dark after 3e7	
	S	T
Mass (g)	3.181	3.060
Average	3.121	
SD	0.0860	
SE	3%	

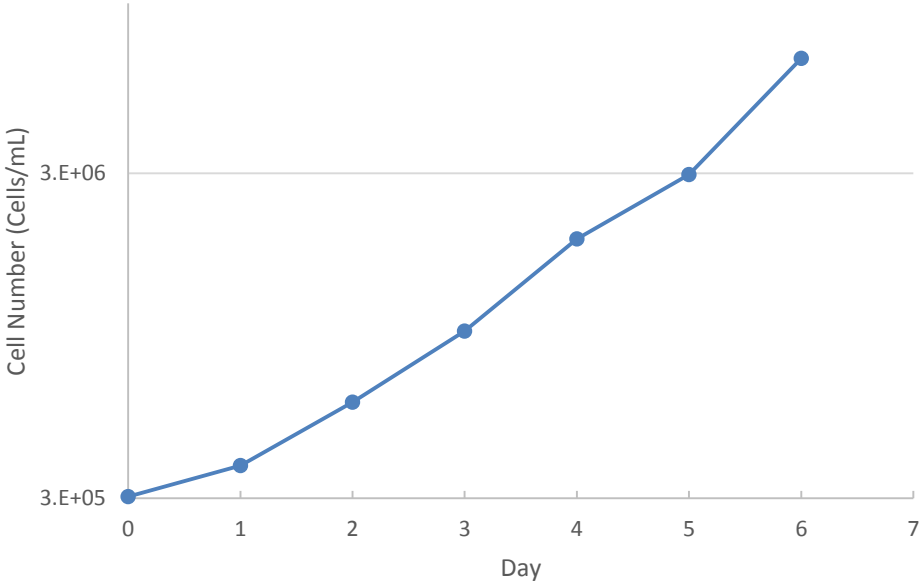

Figure 111. Growth curve of *T.* sp. LCR-Awa9/2 inoculum to start experiment 21.

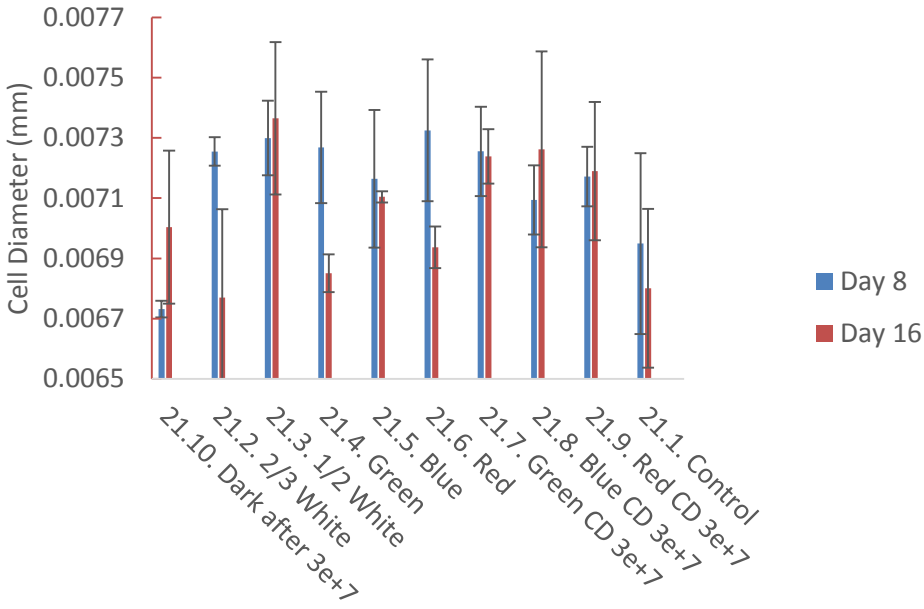

Figure 112. Experiment 21 cell size changing in day 8 and day 16.

Experiment 22

Experimental conditions:
22.1. OHM4N
22.2. 2BBM4N ½ light after 3e7
22.3. 2BBM4N harvested in day 8
22.4. 2BBM1/2N
22.5. 2BBM1/2N harvested after 1e7
22.6. 2BBM4N1/40P
22.7. 2BBM4N1/40P harvested after 1e7
22.8. 2BBM4N
22.9. 2BBM4N $FeSO_4$ + Na_2EDTA
22.10. 2BBM4N without cobalt
22.11. 2BBM4N without cobalt and boron

Table 40. Experiment 22 cell density.

Day	22.1. OHM4N					22.2. 2BBM4N ½ light after 3e7				
	A	B	Average	SD	SE	C	D	Average	SD	SE
0	227500	272500	250000	31820	13%	197500	205000	201250	5303	3%
1	318750	301250	310000	12374	4%	451250	436250	443750	10607	2%
2	602500	656250	629375	38007	6%	873750	806250	840000	47730	6%
3	1267500	1087500	1177500	127279	11%	1462500	1548750	1505625	60988	4%
4	2409500	2210000	2309750	141068	6%	2989500	2268000	2628750	510178	19%
5	5650000	4000000	4825000	1166726	24%	5550000	5425000	5487500	88388	2%
6	8775000	10025000	9400000	883883	9%	11250000	10450000	10850000	565685	5%
7	12600000	13350000	12975000	530330	4%	15650000	14750000	15200000	636396	4%
8	14950000	15450000	15200000	353553	2%	19950000	21800000	20875000	1308148	6%
9	17300000	17950000	17625000	459619	3%	21600000	30950000	26275000	6611448	25%
10	22500000	20900000	21700000	1131371	5%	26625000	35025000	30825000	5939697	19%
11	26100000	24225000	25162500	1325825	5%	25950000	39900000	32925000	9864140	30%
12	28600000	26800000	27700000	1272792	5%	29200000	47600000	38400000	13010765	34%
13	30300000	28500000	29400000	1272792	4%	33100000	54800000	43950000	15344217	35%
14	33875000	31900000	32887500	1396536	4%	36500000	54750000	45625000	12904699	28%

Day	22.3. 2BBM4N harvested in day 8					22.4. 2BBM1/2N				
	E	F	Average	SD	SE	G	H	Average	SD	SE
0	217500	241250	229375	16794	7%	220000	220000	220000	0	0%
1	432500	525000	478750	65407	14%	476250	491250	483750	10607	2%
2	803750	823750	813750	14142	2%	875000	865000	870000	7071	1%
3	1432500	1440000	1436250	5303	0%	1485000	1462500	1473750	15910	1%
4	2360500	2649003	2504752	204002	8%	2521500	2629500	2575500	76368	3%
5	4775000	5600000	5187500	583363	11%	4325000	5225000	4775000	636396	13%
6	10400000	10350000	10375000	35355	0%	8700000	9950000	9325000	883883	9%
7	15780000	14980000	15380000	565685	4%	9975000	11900000	10937500	1361181	12%
8	22850000	19150000	21000000	2616295	12%	12750000	11800000	12275000	671751	5%
9						15900000	14550000	15225000	954594	6%
10						15000000	15500000	15250000	353553	2%
11						15350000	16275000	15812500	654074	4%
12						16800000	17800000	17300000	707107	4%
13						18200000	19900000	19050000	1202082	6%
14						19200000	20150000	19675000	671751	3%
Day	22.5. 2BBM1/2N harvested after 1e7					22.6. 2BBM4N1/40P				
	I	J	Average	SD	SE	K	L	Average	SD	SE
0	217500	205000	211250	8839	4%	193750	210000	201875	11490	6%
1	478750	455000	466875	16794	4%	333750	428750	381250	67175	18%
2	887500	857500	872500	21213	2%	752500	822500	787500	49497	6%
3	1646250	1402500	1524375	172357	11%	1443750	1702500	1573125	182964	12%
4	2456000	2436500	2446250	13789	1%	3033000	3220500	3126750	132583	4%
5	4550000	4750000	4650000	141421	3%	5975000	5600000	5787500	265165	5%
6	8025000	7675000	7850000	247487	3%	9125000	9400000	9262500	194454	2%
7	10600000	8925000	9762500	1184404	12%	10980000	11750000	11365000	544472	5%
8	12750000	10600000	11675000	1520280	13%	12850000	12700000	12775000	106066	1%
9						13850000	15000000	14425000	813173	6%
10						14150000	13850000	14000000	212132	2%
11						14775000	12975000	13875000	1272792	9%
12						15900000	14800000	15350000	777817	5%
13						16800000	18400000	17600000	1131371	6%
14						17600000	18700000	18150000	777817	4%

Day	22.7. 2BBM4N1/40P harvested after 1e7					22.8. 2BBM4N				
	M	N	Average	SD	SE	O	P	Average	SD	SE
0	232500	201250	216875	22097	10%	235000	217500	226250	12374	5%
1	432500	385000	408750	33588	8%	511250	510000	510625	884	0%
2	786250	806250	796250	14142	2%	918750	907500	913125	7955	1%
3	1511250	1627500	1569375	82201	5%	1642500	1518750	1580625	87504	6%
4	2224000	2350000	2287000	89095	4%	2800000	2990000	2895000	134350	5%
5	4375000	4475000	4425000	70711	2%	6550000	5950000	6250000	424264	7%
6	9675000	8525000	9100000	813173	9%	11025000	11350000	11187500	229810	2%
7	11060000	10850000	10955000	148492	1%	22500000	20900000	21700000	1131371	5%
8	12300000	12500000	12400000	141421	1%	28350000	28800000	28575000	318198	1%
9						37150000	38200000	37675000	742462	2%
10						47400000	47250000	47325000	106066	0%
11						56925000	57600000	57262500	477297	1%
12						64900000	64200000	64550000	494975	1%
13						72200000	72700000	72450000	353553	0%
14						86375000	80500000	83437500	4154252	5%
Day	22.9. 2BBM4N FeSO4 + Na2EDTA					22.10. 2BBM4N without cobalt				
	Q	R	Average	SD	SE	S	T	Average	SD	SE
0	208750	201250	205000	5303	3%	231250	193750	212500	26517	12%
1	447500	458750	453125	7955	2%	400000	460000	430000	42426	10%
2	811250	780000	795625	22097	3%	798750	777500	788125	15026	2%
3	1545000	1357500	1451250	132583	9%	1466250	1361250	1413750	74246	5%
4	2693500	2542000	2617750	107127	4%	2602000	2522000	2562000	56569	2%
5	6300000	5450000	5875000	601041	10%	4700000	4950000	4825000	176777	4%
6	11150000	10375000	10762500	548008	5%	9950000	9625000	9787500	229810	2%
7	19250000	18600000	18925000	459619	2%	17250000	16850000	17050000	282843	2%
8	28250000	27800000	28025000	318198	1%	26250000	25800000	26025000	318198	1%
9	38400000	36800000	37600000	1131371	3%	33650000	34050000	33850000	282843	1%
10	45825000	43600000	44712500	1573313	4%	47075000	49600000	48337500	1785445	4%
11	54675000	51900000	53287500	1962221	4%	59025000	61575000	60300000	1803122	3%
12	64800000	58900000	61850000	4171930	7%	70700000	72600000	71650000	1343503	2%
13	74900000	60400000	67650000	10253048	15%	76700000	85800000	81250000	6434672	8%
14	78625000	78625000	78625000	0	0%	77250000	77500000	77375000	176777	0%

Day	22.11. 2BBM4N without cobalt and boron				
	U	V	Average	SD	SE
0	210000	236250	223125	18562	8%
1	418750	401250	410000	12374	3%
2	762500	748750	755625	9723	1%
3	1487500	1308750	1398125	126395	9%
4	2662500	2353000	2507750	218850	9%
5	5125000	5525000	5325000	282843	5%
6	10300000	11200000	10750000	636396	6%
7	19750000	19400000	19575000	247487	1%
8	28520000	29050000	28785000	374767	1%
9	36650000	38050000	37350000	989949	3%
10	49200000	47600000	48400000	1131371	2%
11	61050000	58500000	59775000	1803122	3%
12	70300000	68200000	69250000	1484924	2%
13	80900000	77600000	79250000	2333452	3%
14	86000000	78500000	82250000	5303301	6%

Table 41. Experiment 22 pH change

Culture	22.1. OHM		22.2. 2BBM4N 1/2 Light after 3e7		22.3. 2BBM4N Harvested in day 8		22.4. 2BBM1/2N		22.5. 2BBM1/2N Harvested after 10e7		22.6. 2BBM4N1/40P	
	A	B	C	D	E	F	G	H	I	J	K	L
Final pH	6.84	6.72	7.03	7.35	7.14	6.9	6.44	6.51	6.48	6.58	6.56	6.72
Average	6.78		7.19		7.02		6.475		6.53		6.64	
SD	0.0849		0.2263		0.1697		0.0495		0.0707		0.1131	
SE	1%		3%		2%		1%		1%		2%	
Culture	22.7. 2BBM4N1/40P Harvested after 10e7		22.8. 2BBM4N		22.9. 2BBM4N $FeSO_4$ Na_2EDTA		22.10. 2BBM4N without Cobalt		22.11. 2BBM4N without Cobalt and Boric			
	M	N	O	P	Q	R	S	T	U	V		
Final pH	6.6	6.44	7.36	7.36	7.28	7.32	7.25	7.32	7.3	7.48		
Average	6.52		7.36		7.3		7.285		7.39			
SD	0.1131		0.0000		0.0283		0.0495		0.1273			
SE	2%		0%		0%		1%		2%			

Table 42. Experiment 22 biomass dry weight

Culture	22.1. OHM		22.2. 2BBM4N 1/2 Light after 3e7		22.3. 2BBM4N Harvested in day 8		22.4. 2BBM1/2N		22.5. 2BBM1/2N Harvested after 10e7		22.6. 2BBM4N1/40P	
	A	B	C	D	E	F	G	H	I	J	K	L
Mass (g)	2.401961	2.334314	1.5343137	2.880392	1.948039	1.742157	2.152	2.144	1.69902	1.712745	1.725	1.707843
Average	2.368137255		2.207352941		1.845098039		2.148		1.705882353		1.716	
SD	0.0478		0.9518		0.1456		0.0057		0.0097		0.012	
SE	2%		4%		8%		0%		1%		1%	
Culture	22.7. 2BBM4N1/40P Harvested after 10e7		22.8. 2BBM4N		22.9. 2BBM4N $FeSO_4$ Na_2EDTA		22.10. 2BBM4N without Cobalt		22.11. 2BBM4N without Cobalt and Boric			
	M	N	O	P	Q	R	S	T	U	V		
Mass (g)	1.67451	2.37549	3.912	3.728	4.314706	4.403922	4.103922	4.012745	4.370297	4.246		
Average	2.025		3.82		4.359313725		4.058333333		4.308148515			
SD	0.4957		0.1301		0.0631		0.0645		0.0879			
SE	24%		3%		1%		2%		2%			

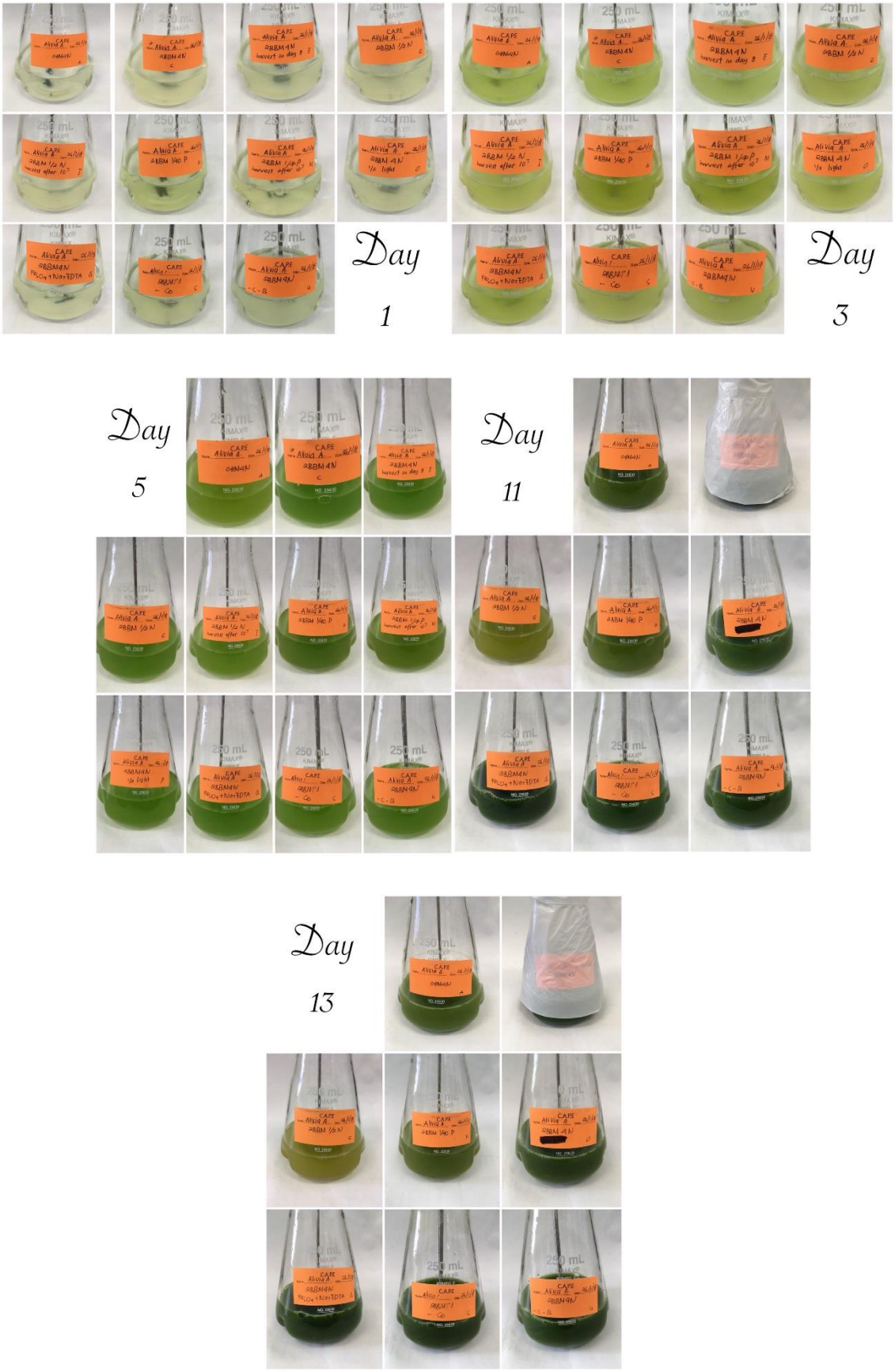

Figure 113. Experiment 22 cultures.

Experiment 23

Experimental conditions:
23.1. BBMA
23.2. 2BBM4N
23.3. BBMA1/2N
23.4. BBMA1/2N harvested on day 10
23.5. BBMA1/2N + 3.5N on day 10
23.6. BBMA1/40P
23.7. BBMA complete starvation on day 10
23.8. BBMA N source: urea+Ni
23.9. BBMA N source: ammonium

Table 43. Experiment 23 cell density.

Day	23.1. Control		Average	SD	SE	23.2. 2BBM4N		Average	SD	SE
	A	B				C	D			
0	218750	256250	237500	26517	11%	202500	227500	215000	17678	8%
1	465000	427500	446250	26517	6%	425000	512500	468750	61872	13%
2	662500	656250	659375	4419	1%	873750	806250	840000	47730	6%
3	1462500	1312500	1387500	106066	8%	1575000	1812500	1693750	167938	10%
4	3562500	2987500	3275000	406586	12%	2662500	2962500	2812500	212132	8%
5	7262500	6975000	7118750	203293	3%	5725000	6125000	5925000	282843	5%
6	11750000	10750000	11250000	707107	6%	11275000	12950000	12112500	1184404	10%
7	21675000	21675000	21675000	0	0%	21675000	19175000	20425000	1767767	9%
8	36675000	31275000	33975000	3818377	11%	28800000	27075000	27937500	1219759	4%
9	42800000	44100000	43450000	919239	2%	32800000	33700000	33250000	636396	2%
10	51700000	53600000	52650000	1343503	3%	35000000	35200000	35100000	141421	0%
11	60200000	58200000	59200000	1414214	2%	40200000	42900000	41550000	1909188	5%
12	67900000	70300000	69100000	1697056	2%	46800000	47500000	47150000	494975	1%
13	73500000	77800000	75650000	3040559	4%	52200000	53300000	52750000	777817	1%
14	77750000	84000000	80875000	4419417	5%	53625000	56875000	55250000	2298097	4%

Day	23.3. 1/2N		Average	SD	SE	23.4. 1/2N harvested in day 10		Average	SD	SE
	E	F				G	H			
0	237500	235000	236250	1768	1%	261250	238750	250000	15910	6%
1	397500	350000	373750	33588	9%	370000	327500	348750	30052	9%
2	803750	823750	813750	14142	2%	875000	865000	870000	7071	1%
3	1775000	1575000	1675000	141421	8%	1750000	1550000	1650000	141421	9%
4	3025000	2887500	2956250	97227	3%	2725000	2900000	2812500	123744	4%
5	5575000	5175000	5375000	282843	5%	5437500	4975000	5206250	327037	6%
6	7250000	6987500	7118750	185616	3%	6825000	6537500	6681250	203293	3%
7	10350000	9325000	9837500	724784	7%	8100000	9150000	8625000	742462	9%
8	11700000	10500000	11100000	848528	8%	11475000	12600000	12037500	795495	7%
9	12100000	12500000	12300000	282843	2%	13000000	11900000	12450000	777817	6%
10	14200000	13800000	14000000	282843	2%	15400000	16500000	15950000	777817	5%
11	13300000	12900000	13100000	282843	2%					
12	13600000	13100000	13350000	353553	3%					
13	12900000	14600000	13750000	1202082	9%					
14	12250000	15625000	13937500	2386485	17%					
Day	23.5. 1/2N + 3.5 N in day 10		Average	SD	SE	23.6. 1/40P		Average	SD	SE
	I	J				K	L			
0	277500	283750	280625	4419	2%	195000	190000	192500	3536	2%
1	382500	430000	406250	33588	8%	282500	302500	292500	14142	5%
2	887500	857500	872500	21213	2%	752500	822500	787500	49497	6%
3	1575000	1325000	1450000	176777	12%	1412500	1137500	1275000	194454	15%
4	2587500	2325000	2456250	185616	8%	2462500	2312500	2387500	106066	4%
5	5375000	4925000	5150000	318198	6%	5075000	4937500	5006250	97227	2%
6	8825000	7500000	8162500	936916	11%	8987500	8675000	8831250	220971	3%
7	9475000	8250000	8862500	866206	10%	10125000	8850000	9487500	901561	10%
8	9775000	10650000	10212500	618718	6%	11775000	10325000	11050000	1025305	9%
9	12500000	13400000	12950000	636396	5%	12200000	11800000	12000000	282843	2%
10	14900000	13900000	14400000	707107	5%	13000000	12100000	12550000	636396	5%
11	16700000	15500000	16100000	848528	5%	12600000	13200000	12900000	424264	3%
12	21300000	18900000	20100000	1697056	8%	13600000	13900000	13750000	212132	2%
13	25800000	23600000	24700000	1555635	6%	14000000	13100000	13550000	636396	5%
14	32500000	29625000	31062500	2032932	7%	14750000	14125000	14437500	441942	3%

Day	23.8. Urea+Ni		Average	SD	SE	23.9. NH4+		Average	SD	SE
	O	P				Q	R			
0	223750	263750	243750	28284	12%	228750	211250	220000	12374	6%
1	297500	315000	306250	12374	4%	372500	395000	383750	15910	4%
2	512500	507500	510000	3536	1%	811250	780000	795625	22097	3%
3	1275000	1500000	1387500	159099	11%	1400000	1237500	1318750	114905	9%
4	2512500	3112500	2812500	424264	15%	2675000	2375000	2525000	212132	8%
5	5825000	6275000	6050000	318198	5%	4825000	4500000	4662500	229810	5%
6	9825000	11750000	10787500	1361181	13%	5900000	6250000	6075000	247487	4%
7	16500000	20725000	18612500	2987526	16%	7950000	8500000	8225000	388909	5%
8	21925000	18000000	19962500	2775394	14%	9825000	9825000	9825000	0	0%
9	27900000	25200000	26550000	1909188	7%	10600000	11900000	11250000	919239	8%
10	36500000	29600000	33050000	4879037	15%	11900000	13500000	12700000	1131371	9%
11	38300000	36100000	37200000	1555635	4%	15200000	18000000	16600000	1979899	12%
12	40100000	42600000	41350000	1767767	4%	18500000	22500000	20500000	2828427	14%
13	41100000	48900000	45000000	5515433	12%	21800000	27000000	24400000	3676955	15%
14	43875000	55750000	49812500	8396893	17%	25375000	31750000	28562500	4507806	16%

Day	23.7. Complete Starvation on day 12		Average	SD	SE
	M	N			
0	218750	218750	218750	0	0%
1	505000	460000	482500	31820	7%
2	786250	806250	796250	14142	2%
3	1425000	1375000	1400000	35355	3%
4	2762500	2475000	2618750	203293	8%
5	5825000	4975000	5400000	601041	11%
6	11250000	8625000	9937500	1856155	19%
7	16875000	13525000	15200000	2368808	16%
8	27225000	22025000	24625000	3676955	15%
9	37600000	29800000	33700000	5515433	16%
10	46800000	38600000	42700000	5798276	14%
11	54800000	46300000	50550000	6010408	12%
12	62400000	54000000	58200000	5939697	10%
13	70125000	62750000	66437500	5214913	8%
14	77375000	69750000	73562500	5391689	7%
15	80250000	78750000	79500000	1060660	1%
16	82625000	86375000	84500000	2651650	3%
17	86750000	90125000	88437500	2386485	3%

Table 44. Experiment 22 pH change

Culture	23.1. Control		23.2. 2BBM4N		23.3. 1/2N		23.4. 1/2N harvested in day 10		23.5. 1/2N + 3.5 N in day 10		23.6. 1/40P	
	A	B	C	D	E	F	G	H	I	J	K	L
Final pH	7.51	8.16	7.46	7.36	6.36	6.31	6.79	6.31	7.39	7.37	6.87	6.7
Average	7.835		7.41		6.335		6.55		7.38		6.785	
SD	0.4596		0.0707		0.0354		0.3394		0.0141		0.1202	
SE	6%		1%		1%		5%		0%		2%	

Culture	23.7. Complete Starvation on day 12		23.8. Urea+Ni		23.9. NH_4^+	
	M	N	O	P	Q	R
Final pH	7.2	7.6	6.99	6.7	6.3	6.13
Average	7.4		6.845		6.215	
SD	0.2828		0.2051		0.1202	
SE	4%		3%		2%	

Table 45. Experiment 22 biomass dry weight

Culture	23.1. Control		23.2. 2BBM4N		23.3. 1/2N		23.4. 1/2N harvested in day 10		23.5. 1/2N + 3.5 N in day 10		23.6. 1/40P	
	A	B	C	D	E	F	G	H	I	J	K	L
Mass (g)	4.6319	4.7967	3.0194	3.5495	2.0741	1.8859	1.6630	1.6600	2.9827	2.6058	2.1281	1.6425
Average	4.71		3.2844		1.98		1.6615		2.7943		1.89	
SD	0.12		0.3748		0.1331		0.0021		0.2665		0.343	
SE	2%		11%		7%		0%		10%		18%	

Culture	23.7. Complete Starvation on day 12		23.8. Urea+Ni		23.9. NH_4^+	
	M	N	O	P	Q	R
Mass (g)	5.8935	6.5833	3.6635	3.7686	1.7177	1.9660
Average	5.8935		3.7160		1.8418	
SD	0.488		0.0744		0.1756	
SE	8%		2%		10%	

Figure 114. Experiment 23 cultures.

Experiment 24

14 days cultivation of two flasks with the same experimental conditions as Experiment 23 gained 9.3×10^{7} cells/mL culture. Below is experimental data from Experiment 24:

Dry biomass production	: 5.6±0.15 g/L
Dry cell weight	: 6×10^{-11} g/cell
Wet cell weight	: 2.9×10^{-10} g/cell
Wet cell mass fraction	: 0.027
Volume fraction of wet algal pellet	: 0.033

Cell size : 0.0087 mm

9.3×10^7 cells/mL culture's cell specific gravity at 23 ˚C : 1.0022
9.3×10^7 cells/mL culture's viscosity at 23 ˚C : 0.973 mPa.s
Supernatant specific gravity at 23 ˚C : 1.0007
Supernatant viscosity at 23 ˚C : 0.940 mPa.s

Using

$$\frac{1}{\rho_{culture}} = \frac{w_{cell}}{\rho_{cell}} + \frac{w_{supernatant}}{\rho_{supernatant}}$$

where w is the mass fraction we can calculate the cell specific gravity to be 1.06.

Below are selected cell images from Experiment 24.